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Growth Kinetics and Constraints Related to Metabolic Diversity and Abundances of Hyperthermophiles in Deep-Sea Hydrothermal Vents

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GROWTH KINETICS AND CONSTRAINTS RELATED TO METABOLIC DIVERSITY AND ABUNDANCES OF HYPERTHERMOPHILES IN DEEP-SEA HYDROTHERMAL VENTS

A Dissertation Presented

By

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DEDICATION

To each youth being told they learn differently

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ABSTRACT

GROWTH KINETICS AND CONSTRAINTS RELATED TO METABOLIC DIVERSITY AND ABUNDANCES OF HYPERTHERMOPHILES IN DEEP-SEA HYDROTHERMAL VENTS

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This dissertation research aims to show that there are deterministic microbial distribution patterns based on quantifiable environmental thresholds by determining and rationalizing the relative abundances of hyperthermophilic methanogens, autotrophic iron(III) oxide reducers, and heterotrophic sulfur reducers within deep-sea hydrothermal vents. Organisms of these metabolisms are predicted to be relatively more abundant in different regions depending on environmental conditions such as reduction potential, organic carbon, and hydrogen availability. The relative abundances of these metabolic groups within samples from the Endeavour Segment and Axial Volcano in the northeastern Pacific Ocean were determined. Iron(III) oxide reducers were detected in nearly all samples while methanogens were generally not present or present in concentrations lower than those of the iron(III) reducers. To determine growth constraints and the effect of hydrogen concentration on hyperthermophilic methanogen growth kinetics, *Methanocaldococcus jannaschii* and two new *Methanocaldococcus* field isolates were grown at varying hydrogen concentrations. The hydrogen-dependent

growth kinetics for all three strains were statistically indistinguishable, exhibiting longer doubling times and lower maximum cell concentrations with decreasing hydrogen concentrations until growth ceased below 17-23 μM. This minimum hydrogen concentration for hyperthermophilic methanogenesis was correlated with field microbiology and fluid geochemistry data from the Endeavour Segment and Axial Volcano. Anomalously high methane concentrations and thermophilic methanogens were only observed in fluid samples where hydrogen concentrations were above this predicted threshold. Aside from anomalous sites, methanogens are predicted to be hydrogen limited, and may rely on hydrogen produced by heterotrophs as suggested by *in situ* sampling and co-culture experiments. Models and kinetic experiments suggest that iron(III) oxide reducers are not hydrogen limited under the same conditions. A *Methanocaldococcus* strain that we isolated from Axial Volcano and used in our hydrogen threshold experiments was bioenergetically modeled over its range of growth temperatures, pH, NaCl concentrations, and NH_4Cl concentrations. Its methane production rates and growth energies were largely constant but increased at superoptimal temperatures and when nitrogen was limiting. The results of this research demonstrate that the rates of and constraints on metabolic processes can be used to predict the distribution and biogeochemical impact of hyperthermophiles in deep-sea hydrothermal vent systems.

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CHAPTER 1

INTRODUCTION

1.1 Objectives and Hypotheses

The central hypothesis of this dissertation is that there are deterministic microbial distribution patterns based on quantifiable thresholds of environmental conditions. The goal is to determine and rationalize the relative abundances of hyperthermophilic autotrophic iron(III) oxide reducers, methanogens, and heterotrophic sulfur reducers within deep-sea hydrothermal vents. The null hypothesis being tested is that these organisms will be evenly distributed, and if a distribution pattern appears that it is purely stochastic. Characterizations of growth constraints, growth rates, and metabolic rates of representative field isolates of *Methanocaldococcus*, *Thermococcaceae*, and *Pyrodictiaceae* aim to reveal the differences between these metabolic types that can correlate microbial abundances and environmental conditions found in the field.

The archaeal distribution patterns and species found by previous studies mentioned in this introduction have led to the following three initial hypotheses:

1) Environmental factors within a hydrothermal vent system, such as reduction potential, organic carbon availability, mineralogy of sulfide deposit, and gas component concentrations of hydrothermal fluid cause incongruous distribution of hyperthermophiles of different metabolisms.

2) The relative abundance of hyperthermophilic heterotrophs, such as *Thermococcales* species, will be greater when associated with macrofauna near the organically rich surface of hydrothermal sulfide deposits than within sulfide interiors, where autotrophic growth will be favored.

3) Autotrophic iron(III) reducers, such as members of Crenarchaeotal order *Desulfurococcales*, are abundant within sulfide interiors, and may be higher in abundance than other hyperthermophilic autotrophs, such as members of the Euryarchaeota order *Methanococcales*.

The background information provided in this introduction on microbial distribution patterns, the microorganisms studied, and deep-sea hydrothermal vents aims to contextualize the significance of these questions being asked and to rationalize the hypotheses being proposed. This introduction intends to provide a framework for this dissertation on determining the metabolic diversity and abundances of hyperthermophiles in deep-sea hydrothermal vents (chap. 2), defining growth kinetics and constraints of hyperthermophilic methanogens so as to relate thresholds of environmental conditions to microbial distribution patterns (chap. 3), and characterizing a hyperthermophilic methanogen in order to model bioenergetics by measuring growth and metabolic rates under varying environmentally relevant conditions (chap. 4).

1.2 Microbial Distribution Patterns

Biogeography is the study of spatial patterns and processes involved in the distribution of biodiversity. This field has been divided since its origins concerning the explanations of exhibited distribution patterns pitting factors such as ecological vs. historical explanations, and environmental determinism vs. random dispersal (O'Malley, 2007). A major goal of ecological biogeography and community ecology is to find explanations for the observed patterns of species abundance in space and time (Chave, 2004). The major opposing theories explaining community assembly, 'determinist' and 'stochastic,' have been a major issue of contention during the 19th century, and continues to be debated today.

Several stochastic models have been proposed, such as Ehrenberg's infusion theory (Ehrenberg, 1838), MacArthur and Wilson's theory of island biogeography (MacArthur & Wilson, 1963; MacArthur & Wilson, 1967), Hubbell's two-scale model (Hubbell, 1997), Bell's neutral model (Bell, 2000), Chave's spatially structured model (Chave & Leigh, 2002), and most notably Hubbell's neutral model (Hubbell, 2001). Hubbell's neutral model contends that ecological drift is usually the best explanation of community structure, and focuses on 'random' events such as dispersal, birth, death and migration. Ecological equivalence of all individual organisms is the fundamental, yet controversial, idea behind this theory (Hubbell, 2006). Hubbell's neutral theory is primarily concerned with species-rich communities (*i.e.* tropical forests, coral reefs) (Chave, 2004), and has been contested in more selective environments which have lower species richness.

The deterministic campaign has earlier roots, but was strongly solidified in 1934 by the Beijerinck and Baas Becking hypothesis that "everything is everywhere, but the environment selects" (Baas Becking, 1934; Beijerinck, 1913). The cosmopolitan nature of this statement does not suggest that there are no biogeographical patterns, but rather that since microbes are limitlessly dispersible, environmental conditions, rather than anything specifically geographical, will determine which microbes will proliferate to great abundance, maintain themselves in low levels, or die off. If the environment is ecologically appropriate, it does not matter where a particular niche is located, the same types of organisms will appear as in other niches with closely comparable environmental conditions. This link between microbes and environmental conditions was expanded to include the idea of "predictive law-like generalizations" that have the potential to explain laboratory and field observations since the presence or absence of certain microbes could be predicted and practically produced by specific environmental conditions (Beijerinck, 1913; O'Malley, 2007). Darwin, who wrote that "there is no place for stochasticity in population biology (Darwin, 1859)," supported this theory, as have many (micro)biologists since its conception. This deterministic viewpoint has developed into more modern theories of niche-assembly (Hutchinson, 1965). Although continuously debated and sometimes contradicted, there has been continuous work for almost a century exposing the direct influence of environmental factors on biotic distribution patterns.

1.2.1 Influence of Environmental Parameters

It has been well demonstrated that the dominant terminal electron acceptor process of a certain zone can be determined by environmental parameters. As terminal

electron acceptors are sequentially depleted, a succession of physiological types occurs based on the geochemistry of the system and thermodynamics of each metabolic reaction. The oxidant that yields the greatest amount of Gibbs free energy is preferentially used. Then, once this oxidant is depleted, the next most efficient oxidant is utilized. Previous studies show that five redox zones can develop sequentially: (1) oxygen reduction, (2) denitrification and Mn(IV) reduction, (3) iron(III) reduction, (4) sulfate reduction, (5) methanogenesis (Abrams *et al.*, 1998). This progression creates characteristic micro-niches. For example, methanogenic and sulfate reducing zones can overlap so that these organisms can occupy the same niche and can grow commensally, while methanogens and iron(III) reducers are generally segregated (Schrenk *et al.*, 2008). By close proximity sampling within a petroleum hydrocarbon plume, repeated oscillations of the typical spatial succession pattern of six metabolic types was observed, with a significant inverse correlation between iron(III) reducers and methanogens (Bekins *et al.*, 1999).

Direct electrochemical measurement of reduction potential has proven very difficult and erroneous due to slow electrode kinetics and natural oscillations of the internal equilibrium, so predictive models have been sought instead. Attempts to model environments based directly on metabolic products have been difficult or unsuccessful because these can be transported conservatively or metabolized by organisms, making it difficult to determine the exact location of that metabolic zone (Bekins *et al.*, 1999). Models that utilize a predictive marker, often an environmental parameter, have shown to be more reliable.

Hydrogen concentration has been proposed and shown to indicate the predominant terminal electron acceptor process within a system (Chapelle *et al.*, 1996;

Chapelle *et al.*, 1997; Lovley & Goodwin, 1988; Lovley *et al.*, 1994). These hydrogen concentration models have been developed based on the thermodynamics of redox processes (Cordruwisch *et al.*, 1988; Hoehler *et al.*, 1998; Jakobsen *et al.*, 1998; Lovley & Goodwin, 1988) and the reported hydrogen concentration threshold values of redox processes (Chapelle *et al.*, 1997; Lu *et al.*, 2001; Ter Meer *et al.*, 1999; Yang & McCarty, 1998). One model predicts and confirms microbial distribution patterns within an aquifer based on threshold hydrogen concentrations: methanogenesis is limited below 7-10 nM H_2; sulfate reduction below 1-1.5 nM H_2; iron(III) reduction below 0.2 nM H_2; and Mn(IV) or nitrate reduction below 0.05 nM H_2 (Lovley & Goodwin, 1988; Lovley *et al.*, 1994). Hydrogen concentrations have been shown repeatedly to be valuable for analyzing the energetics of microbial processes in an environment (Christensen *et al.*, 2000).

1.2.2 Geochemical and Microbial Patterns at Hydrothermal Vents

The potential impact of the environment on microbial communities within hydrothermal vent systems is proposed to be much more intense than other habitats because of the dynamic and steep chemical and thermal gradients (Takai *et al.*, 2001). In an attempt to model microbial metabolisms within a black smoker chimney structure, it was predicted that oxidative metabolisms (Fe, Mn, CH_4, or S oxidation) are thermodynamically favored below 38ºC, while anaerobic metabolisms (SO_4^{2-} or S^o reduction, or methanogenesis) are thermodynamically favored above this temperature (McCollom & Shock, 1997). However, subsequent models have shown how variations in fluid composition, diffusion, mineral precipitation, and advective transport within vent

chimney walls significantly influence the outcome of such models (Tivey, 2004). The differences in intrafield microbial diversity have been primarily attributed to the concentrations and composition of the gas components in the hydrothermal fluids (Takai *et al.*, 2008b). Limitations of these current models include the lack of consideration of microbial processes such as syntrophy, minimal defined metabolic characteristics of organisms at environmentally relevant conditions, and the minimal attention to major (N,P) and minor (Ni, Mo, W) nutrients (Mehta & Baross, 2006; Orphan *et al.*, 2001; Schrenk *et al.*, 2008).

Distributions of the macrofauna of hydrothermal vents have been mapped and the chemical speciation that drives this ecology has been predicted (Luther *et al.*, 2001). A systematic biogeographic study of microbial populations within vent chimneys has not yet been compiled, but a framework has been provided by observations of specific groups of microorganisms and their biomolecular signatures. In 1992, one of the first studies attempting to estimate the extent and nature of microbial diversity within hydrothermal vents measured intact lipid concentrations across a recovered sulfide flange and found wide variation in microbial biomass and community composition, including a shift from ester-linked (bacterial) to ether-linked (archaeal) lipids towards the warmer regions of the sulfide (Hedrick *et al.*, 1992). A method enabling direct cell counts of sulfide chimney subsections also detected variation in microbial biomass and concluded that microbes are very abundant on the surfaces of active sulfides and that their numbers sharply decrease inside the structures (Chevaldonné & Godfroy, 1997). A study later that year combined selective quantitative culturing with whole cell hybridization with probes targeting archaeal and bacterial 16S rDNA to evaluate the community structure within three

chimney samples (Harmsen *et al.*, 1997). Harmsen *et al.*'s study also found an uneven distribution of a diverse microbial community: greatest biomass located in the upper and outer subsamples, a shift towards heterotrophic metabolisms towards the exterior, and a decrease in abundance and morphological diversity towards the interior. A similar distribution pattern was observed in a sulfide sample analyzed with culture and molecular techniques: rRNA dot slot hybridization, quantitative polymerase chain reaction (qPCR), terminal restriction fragment length polymorphism **(**T-RFLP), and 16S rDNA sequencing and clone analysis (Takai *et al.*, 2001). This study also revealed discrete archaeal phylotype communities and molecular signatures of uncultivated archaea. A similar study that incorporated quantitative culturing, qPCR and catalyzed reporter deposition - fluorescent *in situ* hybridization (CARD-FISH) showed a shift from bacteria to archaea towards the interior of a sulfide (Takai *et al.*, 2004a). As part of the Edifice Rex project (Delaney *et al.*, 2001), an unprecedentedly large active hydrothermal metal-sulfide deposit was recovered from the seafloor, divided into discrete mineralogical zones, and analyzed in terms of cell concentrations, lipid content, 16S rRNA sequences, and phylogenetic ratios with FISH targeting Bacteria, Archaea, Eukarya, Euryarchaeota, Crenarchaeota, *Methanococcales* and *Thermococcales* (Schrenk *et al.*, 2003). This study concluded that biomass and diversity was greatest in the center and least in the high temperature interior; *Methanococcales* were a minimal proportion of the biomass reported in this study, while Crenarchaeota were dominant within the interior portion. Several of these microbial studies also performed a basic mineralogical or geochemical analysis of the sulfide samples or its hosted fluids in an attempt to correlate conditions to

microbial distribution (Schrenk *et al.*, 2003; Takai *et al.*, 2001; Takai *et al.*, 2004a; Zhou *et al.*, 2009).

1.2.3 Significance of Microbial Distribution Patterns

If hyperthermophiles within hydrothermal vents are distributed in an incongruous but predictable pattern dictated by environmental chemistry, then hyperthermophiles have great potential as *in situ* tracers. An *in situ* tracer is something already present in the system being studied that can be used to infer the various chemical and physical components of the system. This would be especially useful in studying hydrothermal vents because of the extreme dynamics of the system and their submersion in seawater, which prevent accurate direct measurements. Since hyperthermophiles are not readily found in background seawater, there is increased confidence that detected hyperthermophiles are from the original sample and not from seawater contamination. If the effect of environmental chemistry on representative organisms can be realized, then the presence of these organisms would be indicative of the chemistry of the system. Understanding the effect of environmental chemistry on hyperthermophiles is applicable to the development of *in situ* instruments for long-term deployment to monitor the chemistry and microbiology of deep-sea hydrothermal vents. *In situ* chemical tracers of deep-sea hydrothermal vents may also be indicative of the subsurface biosphere and astrobiological samples. Learning more about the environment and mechanisms of hyperthermophilic metabolisms would lead to a greater understanding of the geological impact these microbial reactions have on a planet or moon and the geological signatures they leave behind.

1.3 Microorganisms of Interest

Hyperthermophiles are those organisms that grow optimally above 80°C (Stetter, 1996). This study involves hyperthermophilic archaea of different metabolisms and their relative abundances in deep-sea hydrothermal vents. Although archaea represent a smaller proportion (1.8-40%) of the total cells associated with some black smoker sulfide chimneys (Harmsen *et al.*, 1997; Hoek *et al.*, 2003; Schrenk *et al.*, 2003; Takai & Horikoshi, 1999), they appear to be important and major components of the diversity of these structures (Takai *et al.*, 2006). The metabolisms of interest in this study are methanogenesis, autotrophic iron(III) oxide reduction, and heterotrophic sulfur reduction. Anaerobic heterotrophs and methanogens are considered ubiquitous among hyperthermophiles in deep-sea hydrothermal vents (Kelley *et al.*, 2002). Recently described autotrophic iron(III) reducers have been suggested to be important members of black smoker chimney communities (Lovley, 2002; Slobodkin *et al.*, 2001; Slobodkin & Wiegel, 1997). These three groups of organisms are of interest because of their contrasting metabolisms in terms of carbon sources, terminal electron acceptors, hydrogen production/consumption, and presumed growth requirements such as oxidation-reduction potential, and hydrogen availability. Based on previous models from low-temperature aquifers and subsurface environments, the abundances of organisms with these three metabolisms should vary with geochemical conditions within a hydrothermal vent system as well (Lovley *et al.*, 1994; McCollom & Shock, 1997).

1.3.1 Archaea

Previously thought of as a subset of the Bacteria called Archaebacteria, the Archaea are now accepted as one of the three domains of life alongside with the Bacteria and the Eukaryotes (Figure 1.1). This clear tripartite division of life, spawned from the work of Carl Woese and colleagues, shows through phylogenetic analysis that the small subunit ribosomal RNA clusters into three distinct domains (Woese & Fox, 1977). Further phylogenetic analysis has divided the Archaea into the Crenarchaeota, the Euryarchaeota, the Nanoarchaeota, and the Korarchaeota. Much more is known about members of the Crenarchaeota and the Euryarchaeota kingdoms than the minimally represented Nanoarchaeota and Korarchaeota. The Crenarchaeota consists of several hyperthermophilic, thermoacidophilic, and mesophilic species while the Euryarchaeota consists of methanogens, extreme halophiles, thermoacidophiles, and hyperthermophiles. *Nanoarchaeum equitans* is the only representative of the Nanoarchaeota, and is an obligate symbiont of the archaeon *Ignicoccus hospitalis* (Huber *et al.*, 2002a). There is one cultured and fully sequenced representative species of the Korarchaeota (Elkins *et al.*, 2008), which is a deeply branching kingdom of the Archaea and evolutionarily is thought to have originated prior to the bifurcation between the Crenarchaeota and the Euryarchaeota (Barns *et al.*, 1996; Elkins *et al.*, 2008). Phenotypically, archaea are no longer thought of exclusively as extremophiles, but as globally ubiquitous representatives of life.

Archaea share some attributes with Bacteria and Eukaryotes, but also have some distinctive features. Bacterial and archaeal ribosomes have similar centrifugation sedimentation rates, namely 70 Svedberg units (70S), while eukaryotic ribosomes have a

sedimentation rate of 80S. Archaea and Bacteria are also similar in that they are exclusively unicellular, similar in size, lack organelles and a nucleus, and have phylogenetically similar proteins for most cellular processes other than DNA and RNA synthesis. In contrast, Archaea are similar to Eukaryotes in that they lack peptidoglycan, contain introns, contain histone-like proteins, have similar RNA polymerases, initiate DNA replication in a similar manner, and initiate tRNA by methionine rather than formylmethionine (Ganoza *et al.*, 2002; Pereira *et al.*, 1997; Zillig, 1991). The Archaea are unique primarily based on the composition of their lipid membranes, which contain methyl-branched isoprenoid alcohols ether-linked to glycerol. The lipid membranes of Bacteria and Eukaryotes are composed of fatty acids ester-linked to glycerol (Tornabene & Langworthy, 1979).

1.3.2 Hyperthermophiles

Hyperthermophiles are heat-loving organisms that have an optimum growth temperature above 80ºC (Stetter, 1999). These organisms have the highest optimal growth and survival temperatures on Earth, as opposed to psychrophiles (0-20ºC growth optimum), mesophiles (20-50ºC growth optimum), and thermophiles (50-80ºC growth optimum). Hyperthermophiles usually cannot grow below 60ºC, although some species can remain viable at suboptimal temperatures for years (Segerer *et al.*, 1993). The microbiology, physiology and biochemistry of hyperthermophiles has been summarized in several reviews (Schönheit & Schäfer, 1995; Stetter, 1996). Hyperthermophiles have been found in 37 different phylogenetic genera: 23 Crenarchaeota, 9 Euryarchaeota, 1 Nanoarchaeota, and 4 Bacteria (see (Holden, 2009) for a review). It is interesting to note

the lack of eukaryotic hyperthermophiles, since the Eukaryotes share all other extremes of life with the Archaea and the Bacteria (Holland & Baross, 2003). Most hyperthermophilic species are archaea, but a few bacterial families contain hyperthermophiles as well: *Aquificaceae*, *Thermotogaceae*, and *Thermodesulfobacteriaceae*. Hyperthermophilic genera with the highest optimal growth temperatures, those ≥ 100ºC, are *Hyperthermus, Pyrolobus, Pyrobaculum, Pyrodictium,* and *Pyrococcus*. All of the hyperthermophiles branch deeply in the phylogenetic tree of life, suggesting that hyperthermophiles are the closest extant ancestors to the last universal common ancestor (LUCA) of life and provide insight into the origin and evolution of life (Figure 1.1).

Hyperthermophiles have been detected in and cultured from various habitats around the world (Segerer *et al.*, 1993). Terrestrial habitats of hyperthermophiles include hot springs, mud-holes, heated soils, solfataric fields, and geothermally heated beaches associated with shallow submarine hydrothermal systems. Hyperthermophilic marine habitats include deep-sea hydrothermal vents, anaerobic hot sediments, submarine fumaroles, and active seamounts. Other habitats of hyperthermophiles include petroleum reservoirs, deep mine shafts, geothermal power plants, acidic hot water drainages and soils, coal refuse piles, and oil-bearing deep geothermally heated soils.

At such high temperatures the tendency is for double-stranded DNA to unzip, proteins to unfold and precipitate, and cell membranes to rupture, but hyperthermophiles employ strategies to maintain the stability of these vital components. DNA is stabilized by a high internal salt concentrations that sustain nucleotides, histone-like proteins that bind to DNA and wrap DNA around them, and reverse gyrase that twists DNA to form

tighter associations (Forterre *et al.*, 1996; Grayling *et al.*, 1996). Hyperthermophiles are also recognized as having superior DNA proofreading and repair mechanisms (DiRuggiero *et al.*, 1997). Proteins are stabilized and kept in solution by a high salt concentration that increases electrostatic interactions and salt bridges, chaperone proteins that repair damaged proteins, proteases that degrade irreparable proteins and a range of unique protein stabilizers including heat shock proteins and intrinsic factors (Petsko, 2001). Cross-linked tetraether lipids defend against cell rupture and their proportion to diether lipids increases with temperature in some organisms or remains consistently high in others (Sprott *et al.*, 1991). The tungsten component of hyperthermophilic key enzymes provides a greater thermal stability than the mesophilic common metal molybdenum (Adams & Kletzen, 1996; Kelley *et al.*, 2001a; Roy *et al.*, 1999).

In volatile environments such as hydrothermal vents, hyperthermophiles are routinely exposed to superoptimal temperatures. A 'heat shock response' to superoptimal temperatures has been studied in a few hyperthermophiles in the laboratory. This is not a permanent adaptation, but rather a transient response that provides tolerance to superoptimal temperatures for a few hours by the production of enzymes, organic solutes and biofilms. There is much uncertainty on the topic, but the current predicted upper temperature limit of growth is 130-150ºC and the upper temperature limit for viability may much higher (Daniel *et al.*, 2004; Deming & Baross, 1993; Holden & Daniel, 2004). Hyperthermophilic extracellular lytic enzymes have been shown to be active outside of the cell at temperatures up to 140°C, which extends the biogenic impact temperature of life beyond the temperature limit of the organism (Holden & Daniel, 2004).

1.3.3 Hyperthermophilic Methanogens

Methanogens are defined as obligate methane producing archaea, but otherwise they are diverse with respect to habitat, growth optima, phylogenetics, and utilizable compounds. Habitats of methanogens include termite hindgut, wet wood of trees, rumen, protozoa, cecum, mammalian large intestine, tundras, landfills, marshes, sediments, rice paddies, sewage sludge digesters, and hydrothermal vents. Their growth optima span from 0°C to near 100°C, from freshwater to hypersaline salt concentrations, and from moderately acidic to pH levels above 9. There are no facultative methanogens. While they may use different starting compounds, the end product of their metabolism is methane via the archaeal version of the acetyl-CoA (or Wood-Ljungdahl) pathway. The methyl coenzyme-A reductase (*mcrA*) gene is a universal functional gene for methanogenesis, which yields very similar phylogenetic clustering to 16S rRNA (Luton *et al.*, 2002). All methanogens are strict anaerobes, often requiring highly reduced (<-330 mV) conditions, partially due to several methanogenesis enzymes being very oxygen sensitive. Methanogens are all extremophiles close to the thermodynamic edge because their low energy metabolism yields <1-2 mol ATP (Deppenmeier & Müller, 2008; Valentine, 2007). Energy conservation is achieved by a chemiosmotic mechanism consisting of Na^+ and H^+ gradients, varying the stoichiometry of ion transduction, differentially expressing isoenzymes, and unique enzymes, cofactors and electron carriers (Deppenmeier & Müller, 2008; Valentine, 2007). Over 30 proteins have been identified as unique to methanogens, which form a monophyletic group exclusive to other archaea (Gao & Gupta, 2007). Hyperthermophilic methanogens, composed of *Methanopyrus*, *Methanothermus*, *Methanotorris* and *Methanocaldococcus* species, have an even larger

energy threshold to overcome, yet with doubling times of ~30 minutes these are some of the fasting growing methanogens.

Methanothermus fervidus and *Methanothermus sociabilis* are terrestrial hyperthermophilic organisms (optimum growth temperatures of 83 and 88°C, respectively) that were isolated from solfataric fields in Iceland (Lauerer *et al.*, 1986; Stetter *et al.*, 1981). Marine hyperthermophilic methanogens from hydrothermal vents include *Methanocaldococcus fervens, Methanocaldococcus indicus, Methanocaldococcus infernus, Methanocaldococcus jannaschii, Methanocaldococcus vulcanius, Methanopyrus kandleri, Methanotorris formicicus, Methanotorris igneus,* and various field isolates of these genera (Burggraf *et al.*, 1990a; Jeanthon *et al.*, 1998; Jeanthon *et al.*, 1999a; Jeanthon *et al.*, 1999b; Jones *et al.*, 1983a; Kurr *et al.*, 1991; L'Haridon *et al.*, 2003; Mehta & Baross, 2006; Takai *et al.*, 2004b). These marine organisms collectively have permissive growth temperatures of 45-110°C with optima ranging from 80-98°C. These hyperthermophiles all grow optimally at circumneutral pH (~6.5). Hydrogen and carbon dioxide can be used by all of these hyperthermophilic organisms to produce methane and in the majority of these species alternatives such as formate, acetate, methanol, or methylamines cannot be utilized. In hydrothermal habitats, hydrogen and carbon-dioxide are common substrates formed from geological processes in the absence of organic matter, so it is logical that isolates from these environments are hydrogenotrophic autotrophs. Hydrogenotrophic hyperthermophilic methanogens are likely to exist in the deep subsurface in a novel environment referred to as HyperSLime: a hydrogen-based hyperthermophilic subsurface lithoautotrophic microbial ecosystem (Chapelle *et al.*, 2002; Nealson *et al.*, 2005; Takai *et al.*, 2004a).

1.3.4 Hyperthermophilic Iron(III) Oxide Reducers

Microbial iron respiration is widespread in terrestrial, marine, and subsurface environments and is suggested to have a significant impact on the geochemistry of these environments (Lovley, 2000a; Lovley, 2000b; Slobodkin *et al.*, 1999). In general, dissimilatory reduction of insoluble iron involves three known mechanisms 1) direct contact with the iron, 2) secretion of iron chelators that solubilize the iron and return it to the cell for reduction without direct contact, and 3) the reduction and release of extracellular electron shuttles for iron reduction without direct contact (Holden *et al.*, 2011). Comparisons of the best understood mesophiles (*Shewanella* and *Geobacter*) with the most studied hyperthermophiles (*Pyrobaculum* and *Geoglobus*) reveal distinct differences in iron reduction mechanisms, suggesting that an unknown alternative is occurring in these and potentially all high temperature iron reducers (Childers & Lovley, 2001; Feinberg & Holden, 2006; Holden & Adams, 2003). Vargas *et al.* suggested that iron(III) reduction is a ubiquitous ancestral ability of all hyperthermophiles because *Pyrobaculum, Pyrodictium, Methanopyrus, Geoglobus, Ferroglobus, Pyrococcus, Methanococcus*, and *Thermotoga* species were capable to some degree of oxidizing hydrogen and reducing iron(III) to iron(II) (Vargas *et al.*, 1998). Iron(III) is available as an electron acceptor in hot microbial ecosystems including the deep hot subsurface (Gold, 1992; Liu *et al.*, 1997), petroleum reservoirs (Greene *et al.*, 1997; Nazina *et al.*, 1995), terrestrial hot springs (Bonch-Osmolovskaya *et al.*, 1999; Brock & Gustafson, 1976), and in deep-sea hydrothermal vents (Jannasch, 1995; Karl, 1995).

The hyperthermophilic iron reducers of deep-sea hydrothermal vents are mainly of the families *Archaeoglobaceae* and *Pyrodictiacea*e. The marine hydrothermal isolate

Ferroglobus placidus was shown to reduce iron(III) in the presence of acetate or aromatics, although it was originally characterized as only being able to reduce nitrate by oxidizing iron(II) or reduce thiosulfate by oxidizing hydrogen (Hafenbradl *et al.*, 1996; Tor *et al.*, 2001; Tor & Lovley, 2001). This is an example of iron(III) expanding metabolic capability, as it was previously unproven that hyperthermophiles could utilize acetate or aromatics or that *F. placidus* could reduce iron(III). *Geoglobus ahangari* and *Geoglobus acetivorans* are obligate iron(III) reducers isolated from hydrothermal vents with optimum growth temperatures of 88ºC and 81ºC, respectively (Kashefi *et al.*, 2002; Slobodkina *et al.*, 2009). *G. ahangari* was the first hyperthermophile isolated with poorly crystalline iron(III) oxide. This environmentally relevant form of iron(III) has been used to isolate novel species and to metabolically expand cultured species of the *Pyrodictiaceae* family (Holden, unpublished; Kashefi & Lovley, 2003; Vargas *et al.*, 1998; Ver Eecke *et al.*, 2009). These *Pyrodictiaceae* species are among the hottest in culture, with optimum temperatures up to 106°C and the ability to survive 121°C autoclaving (Blöchl *et al.*, 1997; Kashefi & Lovley, 2003; Pley *et al.*, 1991; Stetter *et al.*, 1983).

1.3.5 Hyperthermophilic Heterotrophic S° Reducers

Elemental sulfur is present in geothermal environments and is used by hyperthermophiles as a terminal electron acceptor for heterotrophy and chemolithoautrophy. The hyperthermophilic autotrophic genera *Pyrodictium, Thermoproteus, Pyrobaculum,* and *Acidianus* are capable of sulfur reduction via oxidizing hydrogen and producing sulfide. The most extensively studied heterotrophic

sulfur reducers are of the genera *Thermococcus* and *Pyrococcus*, which predominately respire peptides. *Pyrococcus* species, including *P. horikoshii, P. abyssi,* and *P. furiosus,* were isolated from hydrothermal vents or heated marine sediments. *Pyrococcus* species have optimum growth temperatures of 98-100°C and growth permissive and survival temperatures above 100°C (Erauso *et al.*, 1993; Fiala & Stetter, 1986; González *et al.*, 1998; Holden & Baross, 1995). The *Thermococcus* genus contains the highest number of characterized isolates, including the deep-sea hydrothermal vent isolates *T. atlanticus, T. pacificus,* and *T. hydrothermalis* (Godfroy *et al.*, 1997; Marteinsson *et al.*, 1999; Miroshnichenko *et al.*, 1998). *Thermococcus* species grow between 60-100°C with optimum conditions around 85°C. *Thermococcus* species are commonly used as indicators of hot biotypes in the subseafloor and sulfides because their minimum growth temperatures are above 50°C, and they are ubiquitous in hydrothermal systems, utilize a wide range of substrates, grow well in artificial media, and can survive low-temperature oxic conditions (Holden *et al.*, 1998; Kelley *et al.*, 2002; Summit & Baross, 2001)

Peptide respiration is the principle metabolic pathway for these *Thermococcales,* however, growth has been observed in some species on starch, maltose, pyruvate, and cellobiose. At hydrothermal vents, it is presumed that the major source of organic material for these organisms comes from animals and other microbes (Kelley *et al.*, 2002). Hydrogen sulfide, carboxylic acids, and carbon dioxide are the main metabolic products of these reactions. Hydrogen is also formed by hydrogenases that reduce protons, and this product can be inhibitory to growth at high concentrations. Sulfur reduction is preferred over hydrogen production most likely for thermodynamic reasons.

1.3.6 Significance of Organisms

With respect to this study, examining hyperthermophilic methanogens, iron(III) oxide reducers, and S° reducing heterotrophs provides interesting insights into the environmental conditions within deep-sea hydrothermal vents. The comparative abundances of hydrogen producing heterotrophs with hydrogenotrophic autotrophs can yield information on the localization and cycling of carbon and hydrogen within this system. Hyperthermophilic heterotrophs, such as those of the *Thermococcaceae,* are known to be ubiquitous in deep-sea hydrothermal systems (Kelley *et al.*, 2002) and can potentially serve as an environmental tracer of temperature and pH ranges suitable for mildly-acidic to neutral pH hyperthermophiles. Methanogens of the genus *Methanocaldococcus* and iron(III) reducers of the *Pyrodictiaceae* family have similar pH (mildly-acidic to neutral) and temperature (hyperthermophilic) requirements but based on mesophilic analyses are not predicted to be colocalized in the same niche (Bekins *et al.*, 1999; Lovley & Goodwin, 1988; Lovley *et al.*, 1994). One of these autotrophs being relatively more abundant than another might indicate that environmental factors such as reduction potential, hydrogen availability, or mineralogy are preferentially selecting one of these metabolisms.

There are significant reasons to study hyperthermophilic archaea because so much less is known about these high temperature representatives of life than their less extreme counterparts, especially with respect to their physical and chemical constraints and how this relates to their environments. The search for novel archaea and hyperthermophiles has intensified in the last decade for three main reasons 1) the broadening of what life is and under what range of conditions it can exist, 2) the attributes of these organisms have

a great potential to a wide array of industrial purposes, 3) the insights these organisms provide into the origins of life.

The discovery of hyperthermophiles drastically extended the theoretical upper temperature limit of life, which was presumed to be 60-80ºC for a long time (Segerer *et al.*, 1993). The upper temperature limit of life would be the temperature that does not allow the resynthesis of thermostable compounds at a rate sufficiently higher than the rate of their destruction (Stetter *et al.*, 1986). Pressure is related to the upper temperature limit of life because pressures must remain high enough to maintain water in a liquid state. Increased pressures have also been found to have favorable effects on the growth of hyperthermophiles, raising the maximum growth and metabolic temperatures of some and increasing the optimum temperature of others (Holden & Daniel, 2004). Cellular growth and activities have not been conclusively demonstrated at temperatures above 122ºC, but there is no reason to declare this as the ultimate universal upper limit for life (Schrenk *et al.*, 2008; Takai *et al.*, 2008a). Having a good understanding of temperature and other limitations of life is paramount to understanding our planet. Models predicting the biomass of the Earth must have a boundary of habitable space; the upper temperature limit of 110-150°C places a limit on the depth that life can exist to 3-10 km (Gold, 1999; Whitman *et al.*, 1998).

Enzymes of hyperthermophiles have proven valuable to biotechnology and industrial fields. Extracellular enzymes such as amylases, proteases, xylanases, pullulanases, glycotransferases, cellulases, as well as intracellular enzymes such as dehydrogenases, oxidoreductases, and DNA polymerases have been produced either by large scale production or by mesophilic expression (Huber & Stetter, 1998; Schiraldi &

De Rosa, 2002). Hyperthermophilic enzymes show no significant loss of activity at 100ºC and are active at temperatures well above the temperature maxima of the organisms (Huber & Stetter, 1998). Hyperthermophilic temperatures save the cost of cooling and eliminate the problems associated with sterility and viscosity at low temperatures (Huber & Stetter, 1998; Schiraldi & De Rosa, 2002). Industrial design applications of archaea include the production of detergents, sweeteners, and impact resistant resins. Biohydrometallurgy by hyperthermophilic organisms can purify gold, coal, and ore by leaching contaminants such as pyrite (Huber & Stetter, 1998). The unique stable ether linked lipids of archaea have been proven valuable to the medical field in terms of liposome formation, drug delivery, immune response stimulus, and bioelectronics (De Rosa *et al.*, 1994; Gambacorta *et al.*, 1995; Patel & Sprott, 1999). Hyperthermophilic archaea also have positive implications for the environment because they can bioremediate heavy metals such as U, Tc, Cr, Co, Mn and degrade organic pollutants such as sewage sludge, textile waste water, and tire rubber (Huber & Stetter, 1998; Kashefi & Lovley, 2000; Schiraldi & De Rosa, 2002).

Hyperthermophiles represent the deepest branch points and shortest lineages close to the theoretical root of the phylogenetic tree and are thus considered to be the most ancient living organisms and closely related to the postulated primitive first forms of all extant life (Figure 1.1) (Woese, 1987; Woese *et al.*, 1990; Zillig, 1991). Hyperthermophilic 16S rRNA sequence analysis shows that hyperthermophiles are the slowest evolving organisms within the archaeal and bacterial domains, suggesting that a high temperature was the first selective pressure imposed on life (Olsen *et al.*, 1994; Stetter, 1996; Woese *et al.*, 1990). The "Hyperthermophile Eden" hypothesis suggests

that life originated in a hot environment such as a hydrothermal system or a meteor super-heated (~100°C) ocean (Nisbet & Sleep, 2001). The "Hyperthermophilic Noah" hypothesis suggests that the universal ancestor was not necessarily a hyperthermophile, but that hyperthermophiles were part of a diversified population and were the sole survivors of a meteor impact induced heating of the planet, therefore being the last common pre-impact ancestor (Nisbet & Sleep, 2001). In either hypothesis, hydrothermal systems are an important protective environment for these ancient hyperthermophiles once the ocean cooled. The metabolic design of hyperthermophiles provide clues about pre-biotic forms on the early Earth, which are proposed to involve the hydrogen dependent chemistry of transition-metal sulfide catalysts in a hydrothermal setting (Martin *et al.,* 2008). It is suggested that the earliest forms of life evolved from and gained energy similarly to a proto-life of inorganic iron-sulfur membranes capable of oxidizing hydrogen and reducing iron(III) (Lovley, 2002). Once reduced, iron(II) can readily transfer electrons to other electron acceptors, and this may have led to the intracellular incorporation of iron(III)/iron(II) redox couples that are common in more complex modern electron transport pathways (Vargas *et al.*, 1998). Reactions similar to modern day carbon dioxide reduction and the synthesis of acetate and methane from hydrogen and carbon dioxide could have occurred readily on early Earth without the addition of an external energy source such as lightening, which is required for 'Pre-biotic Broth' hypotheses (Martin *et al.*, 2008). If the last common ancestor was a hyperthermophile, then evolution must have converted hyperthermophilic enzymes into counterpart enzymes that function under all of the varying conditions where we find life

today. The study of present day hyperthermophiles provides information of the origin of life and exposes the evolution of enzymes and metabolic pathways.

1.4 Deep-Sea Hydrothermal Vents

The scientific community was astounded by the 1977 discovery of rich unusual fauna whose existence relied solely on chemosynthetic microorganisms fueled in part by the products of magmatic degassing and water-rock reactions (Kelley *et al.*, 2002). Deep-sea hydrothermal vents are found at tectonically active regions of the world where geothermally heated fluids are emitted from below the seafloor (Figure 1.2 and Figure 1.3). They are surficial expressions of planetary-scale processes of ocean crust formation, plate separation and collision, and global heat loss (Van Dover, 2000). At seafloor spreading centers, oceanic plates are pulled apart by tectonic forces as hot rock from deep in the Earth rises to form new crust and its melt feeds hydrothermal systems. The most studied setting of hydrothermal vents is along mid-ocean ridges, a >75,000 km tectonic spreading center girdling the globe like seams on a baseball (Figure 1.2) (Van Dover, 2000). The newly formed porous crust is minimally sedimented so that fluid circulation occurs readily. Twenty cubic kilometers of ocean crust is accreted (and subducted) annually, and the ocean crust is recycled on average every 61 million years (Schrenk *et al.*, 2010). Back-arc basins form behind volcanic arcs along active plate margins where ocean crust is undergoing subduction beneath a continental plate. During subduction, rocks and sediments melt, forming magmas, and ultimately hydrothermal

systems. It is estimated that there are over 20,000 km of volcanic and back-arc systems globally (Schrenk *et al.*, 2010). Seamounts are found along tectonic spreading centers as well as off axis and have sufficient heat and porosity to drive hydrothermal convection.

In all of these systems, cold oxic seawater circulates down to a hot magma lens where high-temperature fluid-rock reactions create highly reduced, mineral-rich, buoyant fluids that discharge upwards (Figure 1.3). This mineral-rich hydrothermal fluid is in disequilibrium with the surrounding seawater, so that upon contact mineral precipitation occurs and metal sulfide deposits are formed. The measured diversity of hydrothermal fluid composition reflects variation in pressure, temperature, rock composition, brine presence, and the degree of phase separation (Butterfield *et al.*, 1994). During phase separation, elements are fractionated between the liquid (ionic species) and vapor (volatile species) phases. The composition and structure of the sulfide deposits vary globally from subseafloor deposition, simple columnar chimneys, white smokers, to complex chimneys with inner conduits yielding multiple edifices (Van Dover, 2000). Within sulfide deposits there are different mineralogical zones and steep temperature and geochemical gradients, such as a >350°C change in temperature across distances as small as 1-3 cm (Figure 1.3) (Takai *et al.*, 2006). Diffuse fluids represent end-member hydrothermal fluids (200-350°C) that have undergone dilution with cold seawater below the surface or within a sulfide structure. Diffuse flow may issue from porous surfaces of active sulfide deposits or directly from fissures and cracks in the seafloor or lava. Although it varies greatly between fields, diffuse flow is a significant proportion of venting at deep-sea hydrothermal vents and is estimated to be responsible for ~50% for the heat flux emanating from crusts at mid-ocean ridges (Huber & Holden, 2008). The

proportion to which diffuse fluid is diluted can be calculated from the concentration of a non-reactive compound that is present in end-member hydrothermal fluid or seawater but not both, such as magnesium, silica, or helium. Diffuse fluid composition is the result of the degree of dilution, the time between dilution and venting, and inorganic and microbially mediated chemical reactions occurring though the upflow-mixing (Butterfield *et al.*, 1997).

Hydrothermal vents are a vivacious haven of diverse life in stark contrast to the relatively barren seafloor. The first microscopic analyses of deep-sea hydrothermal vent water samples estimated $10^8 - 10^9$ cells ml^{-1}, which is four orders of magnitude greater than bacterial concentrations in background seawater (Corliss *et al.*, 1979). Later microbial analysis values have been lower, but have continuously confirmed that hydrothermal vents, and the deep biosphere feeding them, are rich habitats supportive of microbial growth. Dissolved ions and solutes (NH_4^+, Fe^{2+}, Mn^{2+}, organic compounds) and volcanic gases (H_2S, H_2, CO, CH_4) can support primary and secondary microbial production (Karl, 1995). Hydrothermal vent microbial habitats include fluids, sulfide edifices, sediments, microbial mats, animal symbionts, and the surfaces of animals or rocks. The microbial ecology of deep-sea vents has been reviewed extensively (Jannasch, 1995; Jeanthon, 2000; Kelley *et al.*, 2002; Schrenk *et al.*, 2008; Takai *et al.*, 2006; Tivey, 2004). Deep-sea hydrothermal vents represent the most physically and chemically diverse habitats on Earth, which is evident in the microbial diversity spanning from psychrophiles to hyperthermophiles, from obligate chemolithoautotrophs to heterotrophs, and from aerobes to anaerobes (Takai *et al.*, 2006). As one would logically expect, there are evident microbial distribution patterns of these very diverse microbes

within these multifarious habitats, where microbes best suitable for a certain environment are relatively more abundant.

1.4.1 The Endeavour Segment of the Juan de Fuca Ridge

The Endeavour Segment is located on the northern portion of the Juan de Fuca Ridge, a 500 km mid-ocean ridge spreading center located 2,200 m deep off the coast of Washington, Oregon and British Columbia (Figure 1.2A and Figure 1.4A). Due to its proximity to North America, vents along the Juan de Fuca Ridge are some of the most intensively studied on Earth (Fornari & Embley, 1995). The hydrothermal venting at Endeavour is considered the most vigorous and of the highest density of studied vents (Butterfield *et al.*, 1994; Kelley *et al.*, 2002). There were large series of earthquakes in the system in June 1999 and January 2000 (Bohnenstiehl *et al.*, 2004). There are uncertainties as to whether these earthquakes were magmatic or purely tectonic, but they did not create a new lava flow (Johnson *et al.*, 2000; Lilley *et al.*, 2003). These earthquakes may maintain high crustal permeability, which facilitates flow along the fault networks (Johnson *et al.*, 2000; Kelley *et al.*, 2001b). Endeavour is thought of as a geologically "mature" system that apparently lacks recent volcanism (Butterfield *et al.*, 1994). Dating has estimated Endeavour sulfide material to be >200 years old and basalts to be 5,800-8,100 years old (Butterfield *et al.*, 1994; Lilley *et al.*, 1993).

There are currently five major hydrothermal vent fields of the Endeavour Segment that are regularly spaced within a ~150m deep axial valley: Sasquatch, Salty Dawg, High Rise, Main Endeavour, and Mothra (listed from north to south) (Figure 1.4C) (Kelley *et al.*, 2002). The spacing of these high-temperature vent fields and the

presence of diffuse low-temperature flow along-axes between these fields suggest a long-term pattern of stable, nested, subsurface hydrothermal circulation of axial downwelling and upwelling fluids (Kelley *et al.*, 2002; Seyfried *et al.*, 2003; Van Ark *et al.*, 2007). There are dramatic changes in the style, intensity, and thermal-chemical characteristics of venting between the different fields. The diversity seen throughout this region may be indicative of the chronological stages of hydrothermal vent systems.

Sasquatch and Salty Dawg are the northern most fields that are limited to smaller venting regions, fewer sulfides, and slightly lower temperatures. Sasquatch was discovered 6 km north of Main Endeavour in 2000. It extends for >200 m but active venting is limited to a few small structures in the northern 25 m portion of the field where fluids reach 284ºC. Extinct massive sulfides continuously cover the 200 m Sasquatch field. It has been suggested that the active sulfides are a rejuvenation of a previously extinct field by the seismic activity in 1999-2000 (Kelley *et al.*, 2001a). Salty Dawg has a few large sulfide edifices that reach 25 m and release 296ºC fluid, but diffuse flow dominates. As in Sasquatch, there are abundant extinct sulfides throughout the field. Because of this and the fluid chemistry of the system, it has been suggested that this field is waning towards extinction (Kelley *et al.*, 2001a). There are also examples of completely extinct fields in this region; east of the Main Endeavour field is an extensive extinct field that hosts more extinct massive sulfide deposits than active deposits currently in the Main Endeavour field (Kelley *et al.*, 2001a).

The High Rise and Main Endeavour fields have massive, steep-sided, sulfide-sulfate-silica deposits with characteristic flanges. Flanges are overhanging ledges protruding horizontally from the sulfide edifice, and can range from a few centimeters to

seven meters (Figure 1.3). They are formed by venting sideways through the outer walls of the growing sulfide deposit, possibly due to the blockage of an upward flowing channel. Pools of hot (375ºC) water are trapped by the concave lower flange surfaces so that a reflective, shimmering interface is formed with seawater (Delaney *et al.*, 1992). The buoyant fluids diffuse upward through the flanges creating a stratified heated environment suitable for microbes. Temperatures on the tops of active flanges are typically 30-50ºC, but have been recorded to be as high as 150ºC. There are at least four different styles of hydrothermal output: typical black smoker activity, underflow from a flange, diffuse flow from the tops of flanges, and diffuse flow from basaltic substrates (lower temperature). Flange development may play several integral roles in the formation of the large sulfide structures (Delaney *et al.*, 1992). Their horizontal expansion increases the diameter of the structure providing stability for further vertical growth, which is accomplished by the accumulation and incorporation of black smoker debris. Flanges also enhance depositional efficiency because fluids percolating vertically will deposit greater proportions of the dissolved mineral load (Delaney *et al.*, 1992). These massive multi-flanged sulfide structures range from 1,000-10,000 m^3 of sulfide material, whereas the most active vent field of Axial Volcano, another venting site of the Juan de Fuca Ridge, hosts sulfide structures of ~100 m^3 (Butterfield *et al.*, 1994).

The characteristics of sulfide deposits in the High Rise Field include focused hydrothermal flow (up to 343ºC), rapid vertical growth yielding high height to width ratios (up to 3:1), and the presence of larger more mature flanges on the lower portion and smaller younger flanges towards the top of the structures (Robigou *et al.*, 1993). There are at least ten active massive sulfide deposits of this nature. At the time of its

discovery, the sulfide structure named ‘Godzilla’ was the tallest sulfide structure ever described, but it has since fallen over and decreased in size. It was 45 m from trunk to crown with 15-16 tiers of flanges vertically decreasing in size from 7 m. In 2008, the tallest structure in all of the Endeavour Segment was reported to be 28 m tall just south of the Godzilla vent (Clague *et al.*, 2008). Ventnor is a distinguishable sulfide deposit in that it lacks a trunk and is made up entirely of flanges, leading to a mushroom-like appearance.

The Main Endeavour field encompasses a 200 × 400 m field and at least 21 activity venting multi-flanged sulfide edifices, which contain at least 100 vigorously venting black smokers (Kelley *et al.*, 2001a). Sulfide structures are commonly >30 m in diameter and ~20 m in height and discharge vent fluid from multiple black smoker chimneys and numerous flanges (Figure 1.4E). Maximum venting temperatures are 375ºC associated with the smaller structures of the field; the larger structures are typically 20-30ºC less. The fluid chemistry measured at Main Endeavour is very unique for an unsedimented hydrothermal system. Main Endeavour has anomalously high methane and ammonia concentrations that cannot be explained by pure phase separation. It is presumed that these elevated levels are due to thermal decomposition of organic material (Lilley *et al.*, 1993). End-member chlorinity content decreases systematically and incrementally from the northern sulfide structures (~505 mmol/kg / 94% of seawater at Hulk) to the southern sulfide structures (~225 mmol/kg / 47% of seawater at Peanut), which is suggestive of sediments, brine, and phase separation (Butterfield *et al.*, 1994). Although Main Endeavour appears sediment-free, it shows many chemical characteristics of sediment covered and back arc-systems, suggesting there may be buried sediment.

The Mothra field is the largest field, extending over 500 m in length. The sulfide structures of Mothra are in sharp contrast to those of the other fields in that they lack flanges and black smokers are rare (Figure 1.4F) (Kelley *et al.*, 2001b). There are at least five actively venting sulfide clusters composed of multiple steep-sided pinnacles that rise up 20 m. The outer surface of these sulfide complexes are bathed in a shimmering mixture of seawater and hydrothermal fluid percolating outward through the sulfide walls (< 200ºC). The largest of the five clusters is the Faulty Towers complex, which is a 25 m long zone of pinnacles that coalesce to form an array of 7 m high massive sulfide base. In 1997 and 1998, the 'Edifice Rex' project successfully recovered four large (~1800 kg and ~2 m each) and pristine sections of active sulfide chimneys from Mothra, to investigate relationships among the distribution of microbes and the thermal, chemical, and mineralogical gradients (Delaney *et al.*, 2001).

As stated previously, the Endeavour Segment has been extensively studied for decades, inclusive of numerous microbiology examinations. It has been designated by the National Science Foundation's Ridge 2000 Program as one of three Integrated Study Sites, which promotes numerous field expeditions to understand geological, biological, and chemical linkages. Microbes have been broadly detected with culture dependent (Deming & Baross, 1993; Summit & Baross, 2001; Ver Eecke *et al.*, 2009) and molecular techniques (Schrenk *et al.*, 2003; Straube *et al.*, 1990; Wang *et al.*, 2009; Zhou *et al.*, 2009) in various sample types.

1.4.2. Axial Volcano

In 1983, hydrothermal venting was discovered at the intersection of the Cobb-Eickelberg Seamount Chain and the Juan de Fuca Ridge (Embley *et al.*, 1990) and named Axial Seamount (or Axial Volcano) because it dominates the axis of the Juan de Fuca Ridge (Hammond, 1990). Axial Volcano is the most massive active volcano feature found along the ridge axes of the northeastern Pacific Ocean (Fornari & Embley, 1995). The position of Axial Volcano near (or at) the spreading center leads to early stage rift zones (Embley *et al.*, 1990). Axial Volcano is a very seismically active young volcano, the youngest of the Cobb-Eickelberg Seamount Chain (Dziak & Fox, 1999; Embley *et al.*, 1990). The summit of the volcano is relatively shallow, ~1,500 m. The ~3 × 8 km caldera of the volcano has an unusual rectangular shape, which contrasts near-circular forms of similarly sized volcanoes. The caldera wall is breached on the southern end leaving a north/northwest horseshoe shape. Hydrothermal venting is generally found along the axial caldera walls and its rift zones, which extend both north and south from the summit plateau (Figure 1.4B). Diffuse low-temperature venting associated with young volcanism dominates Axial Volcano (Fornari & Embley, 1995).

One of the most active vent fields is the Axial Seamount Hydrothermal Expeditions (ASHES) field, consisting of a 1,200 × 200 m wide area parallel to, and within a few hundred meters of, the southwest wall of the caldera (Hammond, 1990). There is a 100 m diameter area in the northern end characterized by high-temperature (330°C) venting and large (5 m) sulfide chimneys (Figure 1.4B,D) (Embley *et al.*, 1990; Hammond, 1990). Diffuse venting (< 35°C) extends intermittently from this high-temperature region for 200 m northward, and 1,000 m southward, but the only known

sulfide chimneys are within the high-temperature region (Hammond, 1990). ASHES, unlike the other major vent fields of Axial Volcano, does not appear to be associated with a distinct geological feature (Embley *et al.*, 1990).

The vents of the Canadian American Seamount Expedition (CASM) field lie along or near the prominent fissure that extends southwest from the northern caldera rim (Embley *et al.*, 1990). Low-temperature venting (4-7°C) is pervasive along the fissure but hydrothermal precipitants are essentially absent (CASM, 1985). Several large sulfide chimneys are found east and west of the northern part of the fissure with a maximum height of 12 m and a maximum temperature of 35°C (CASM, 1985).

The South Rift Zone (SRZ) exhibits a discontinuous band of low-temperature venting over 4 km extending southward from the breeched southeastern caldera wall (Embley *et al.*, 1990). Axial Gardens is located off-set northward from the caldera wall within collapse pits. Here it is suggested that a considerable amount of warm water flows horizontally through subseafloor passages(Embley *et al.*, 1990), which causes the purely diffusely venting sites such as Marker 113. It is over a portion of this SRZ that the youngest lava from the 1998 eruption flowed.

On January 25, 1998, an intense swarm of earthquakes started at the summit of Axial Volcano (Dziak & Fox, 1999). The earthquake swarm lasted 11 days and included 8,247 detected earthquakes, activity exceeded 120 events/h and reached acoustic magnitudes of 228 dB (Dziak & Fox, 1999). The estimated volume of the lava erupted is 18-76 $\times 10^6$ m^3 for the extrusion and 100-150 $\times 10^6$ m^3 for the intrusion (Embley *et al.*, 1990). This lava flowed over the southeastern part of the caldera and caused dramatic changes to the hydrothermal systems including the partial or total burial of pre-existing

vents, colonization of new lava, and a subsurface microbial bloom (Embley *et al.*, 1999). This bloom was manifested by a 'snow-blower vent" with fluid filled with white flocculated material. The path of the 1998 lava flow has been mapped to show the burial of hydrothermal vents along the southeastern caldera wall, and the survival of vents at the southern caldera wall and Axial Gardens (Figure 1.4B) (Butterfield *et al.*, 2004). Two vents in this SRZ were sampled for fluids before and after the 1998 eruption (Butterfield *et al.*, 2004): Marker 113 at Axial Gardens, which was not covered by new lava, was sampled in 1990, following the eruption in July 1998, and has subsequently been sampled nearly every year since 1998. Another vent along the southeastern wall, which was covered by new lava and then named Marker 33 was first sampled in 1997 and likewise has been sampled nearly every year since then. Axial Volcano continues to be of interest as a research site because of the potential for relatively frequent volcanic intrusions and eruptions with consequent perturbations in the hydrothermal system (Fornari & Embley, 1995).

There is evidence of significant amount of microbes and microbial activity within the fluids of Axial Volcano (Butterfield *et al.*, 2004). Average cell counts are elevated over ambient seawater in all fluids except those which may be too hot for microbes too survive (190-315°C). Fluid chemistry suggests that the electron acceptors available to microbes would be oxygen, nitrate, sulfate, and ferric iron, while hydrogen, hydrogen sulfide, and organic carbon would be available energy sources. Low-temperature fluid chemistry anomalies suggest net microbial production of methane, ammonia, and sulfur; oxidation of hydrogen sulfide; reduction of nitrate; metal leaching, and increases in fluid alkalinity. Methane is a particularly interesting bio-tracer because methane is not a major

component of magmatic gas at Axial Volcano but methane concentrations are 150-330 µmol/kg above estimates based on conserved mixing between seawater and pure hydrothermal fluid. Methanogens and methanogenesis tracers have been detected at Axial Volcano, and Marker 113 seems to be a hot-spot for this metabolism (Huber *et al.*, 2009; Mehta & Baross, 2006).

1.4.3 Significance of Deep-Sea Hydrothermal Vents

The discovery of deep-sea hydrothermal vents, a rich and diverse habitat receiving no surface light, was a dramatic discovery. This directly led to another discovery of even more significance: that there is a deep subsurface biosphere of vast proportions. Based on the assumed porosity of the habitable volume of the subsurface, the subsurface biomass may be 2×10^{16} tons, which would exceed that of the terrestrial surface (Gold, 1992; Gold, 1999). Based on averages and extrapolations of environmental cell concentrations and the habitable volume of the subsurface, the calculated biomass and total carbon of the subsurface is 4-6 x 10^{30} cells (30-50% of Earth) and 350-550 $\times 10^{15}$ g (60-100% of plants) (Whitman *et al.*, 1998). Although direct measurements and estimates remain in their infancy, it is widely accepted that the marine subsurface constitutes one of the largest and most widespread reservoirs of biomass on Earth and that the activities of the inhabiting microbes have profound global implications. Microbial studies have shown that the subsurface is capable of sustaining life, as shown by direct (Juniper *et al.*, 2001; Newberry *et al.*, 2004; Sorensen & Teske, 2006) and indirect (Holden *et al.*, 1998; Huber *et al.*, 2002b; Summit & Baross, 2001) measurements. Recent studies have shown that the microbes residing within the

subsurface are metabolically active (Roussel *et al.*, 2008; Teske, 2005). Several, but not all, studies have found the habitat dominated by archaea (Biddle *et al.*, 2006; Lipp *et al.*, 2008). Often phylogenetic analysis of the population reveals phylotypes belonging to previously unknown and uncultivated groups (Sorensen *et al.*, 2004; Sorensen & Teske, 2006), which expands our knowledge of the phylogenetic diversity of the planet but does not help understand the physiological properties of the organisms. Microorganisms' chemical exchanges with the crust may considerably affect the global biogeochemical cycles of carbon and other elements. Subseafloor microbes produce carbon dioxide and methane and liberate key elements including nitrogen, iron, sulfur, and phosphorous from the sediments. The circulating fluids take these nutrients back up into the ocean where they support biomass. The subsurface can be studied by directly drilling core samples, or by collecting samples that are being discharged from the system. Drilling, although the most direct method, can be expensive, dangerous, and inconclusive due to contamination. These problems are reduced, but not eliminated, by going as close to the subsurface biosphere as possible, the mid-ocean ridge system, and examining deep-sea hydrothermal vents as an outcrop of the subsurface. There will always be a connection between hydrothermal vents and their enriching subsurface, as indicated by hyperthermophiles present in low-temperature sediment hosted diffuse fluids, and this connection is magnified during and following tectonic events such as dike injections. Currently, the subsurface is a largely untapped ecosystem and natural resource. The subseafloor is potentially the largest ecosystem on Earth, but it is also the most difficult to access. Mars has been mapped to a higher resolution than Earth's seafloor (Schrenk *et al.*, 2008). The microbiology of the marine subseafloor is just now becoming a target of exploration, the

first scientific drilling mission wholly dedicated to this biosphere occurred only in 2002 (Marscarelli, 2009). There are so many questions about the subsurface that remain unanswered: What kinds of organisms inhabit it? What are the primary producers? How homogenous or connected is it? What are the dimensions? What is the total biomass? What is the carbon productivity? What is the cycling time? How do the microbes affect or how are they affected by chemical fluxes and mineralogy? Through an interdisciplinary evaluation of hyperthermophiles of deep-sea hydrothermal vents, correlations and quantitative models will develop to shed light on some of these globally important questions concerning the subsurface – the "last frontier" of Earth.

The discovery and subsequent studies of the surface independent deep-sea hydrothermal vents has had enormous impacts on philosophically engaging issues of science: the origin of life and the prospect for extraterrestrial life. Supported hypotheses centered on hydrothermal environments being ancient and impervious with available energy, volatiles, and organics have proposed hydrothermal environments as the most probable site for the origin and evolution of life on Earth (Baross & Hoffman, 1985; Corliss *et al.*, 1981; Holm & Andersson, 2005; Nisbet & Sleep, 2001). It is predicted that the origins of life occurred 3.5-3.8 Ga (Schidlowski *et al.*, 1988). Hydrothermal activity and circulation is a fundamental process that has existed as soon as Earth had an appreciable amount of liquid water, and it is proposed to have been even more pervasive at the proposed period of life's origins due to higher global heat fluxes (Martin *et al.*, 2008). Deep hydrothermal environments are considered among the steadiest on the planet, and have remained constant in terms of temperature, pressure, and chemical surroundings for geologically long periods of time (Gold, 1992). At the time of life's

origins, Earth was much more violent and turbulent than today; early life was subjected to "impact frustration," where asteroid impacts may have created sterilizing conditions across most of the planet (Sleep *et al.*, 1989). Deep hydrothermal environments, especially the subsurface, hypothetically served as safe havens from killer impacts. Besides being an optimal place for life to survive, the fact that energy and the elements of life are pervasive in hydrothermal systems makes it a logical site of the origin of pre-biotic and biotic life forms. The abiotic geophysical processes occurring in deep hydrothermal systems flush the system with continuous supplies of volatiles including hydrogen and carbon dioxide. Thermodynamic models have favorably predicted protometabolic chemistry arising from these thermally evolved fluids (Shock, 1992). This availability of life constituents contrasts the 'Pre-biotic Broth' theory of the early/mid 1900s, which requires the input of massive energy such as lightning to generate such necessary components (Haldane, 1929; Miller, 1953; Oparin, 1952). The mineral-catalyzed pre-biotic reactions could have synthesized biochemically relevant organic molecules that evolved to harness more of the energy available within the system, and eventually evolved into what we would designate to be a chemoautotrophic cell. There probably was not a definite beginning to life, but rather a step-by-step route towards complexity, where no single step can be considered *the* step that demarcates living from non-living matter.

Life supported by hydrothermal activity has broadened our knowledge of what life can be and where it can exist, even to other planetary bodies. Earth is no longer thought of as having the exclusive location supportive of life based on solar radiation intensity, because light derived energy is no longer a prerequisite for life. It is entirely

possible that the photosynthetic based surficial life on Earth may be a very obscure branch of life adapted to very rare circumstances (Gold, 1992). Life adapted to inhabit hydrothermally active environments may be pervasive throughout the solar system since many planetary bodies have equally suitable conditions analogous to early Earth and current hydrothermal systems. Europa is the sixth moon of the planet Jupiter, and the smallest of its four Galilean satellites. The surface of Europa is a smooth water-ice shell with two distinct topographical features: red/grey hills thought to be from cryovolcanic eruptions and crisscrossed cracks presumed to be due to ice tectonic activity. Models predict that heat production from tidal flexing and radiogenic decay would produce a liquid ocean beneath the ice shell, which would house hydrothermal activity resembling deep-sea hydrothermal vents on Earth (Chyba & Phillips, 2007). Enceladus is the sixth-largest moon of Saturn and also has a water-ice shell, minimal impact craters, and evidence of cryovolcanism, geologic activity, an internal heat source and a global liquid ocean. A water-rich plume venting from a pressurized subsurface chamber on the moon's south polar region has provided the most compelling evidence for this site harboring a habitable environment (Hansen *et al.*, 2006). Moons such as these that are further away from the sun can serve as models for the early Earth of life's origins, when the sun was presumed to be ~25% less luminous (Chyba & Phillips, 2007). 'Extraterrestrial' life may not have to be within an orbiting planetary body, but may reside on the interior of cosmic particles, as proposed in the meteor ALH84001 (McKay *et al.*, 1996). The hypothesis of 'Panspermia' proposes that living material can be transported through space over astronomical distances. This hypothesis is realized only in the discovery of subsurface life, because unless the living organisms were deep inside

the rock they would not be shielded from cosmic rays of space (Gold, 1992). It is interesting to note the identical nature of all life on this planet and wonder about properties of life on other planetary bodies since these commonalities need not be so if distinct evolutionary branches of life originated. The hypotheses concerning the origin of life on this planet and the possible habitation of other planetary bodies cannot be tested by direct experimentation because we cannot reproduce the unknown environments with certainty. Studying the analogous environments and organisms of deep-sea hydrothermal vents and the deep subsurface biosphere will expand our knowledge on these engaging questions.

1.5 Summary and Research Approach

This dissertation explores the central hypothesis that there are deterministic microbial distribution patterns based on quantifiable thresholds of environmental conditions. This work aims to resolve and reason the relative abundance of organisms in a system based on in-depth characterizations of growth constraints, growth rates, and metabolic rates of representative organisms. First, the relative abundances of hyperthermophilic autotrophic iron reducers, methanogens and heterotrophs within deep-sea hydrothermal vents must be measured. Representative field isolates of *Methanocaldococcus, Pyrodictiaceae,* and *Thermococcaceae* must then be isolated and characterized, to gain full understanding of their growth constraints and their exhibited growth and metabolic rates under varying environmentally relevant conditions.

Measuring growth and metabolic rates simultaneously exposes additional information about the apparent effect of conditions on the bioenergetic requirements and the compensation abilities of an organism.

Integrated interdisciplinary laboratory and field data move to model deep-sea hydrothermal vents by correlating quantified growth constraints of field isolates with environmental conditions and the relative abundances of similar organisms. The development of quantitative models through interdisciplinary experimentation is a recognized goal of hydrothermal vent research, and is crucial to understanding cycling regimes of this complex system. Compilations of characterization data of this approach would further develop models predicting microbial distribution patterns, flux, and cycling within hydrothermal vent systems and the subsurface biosphere.

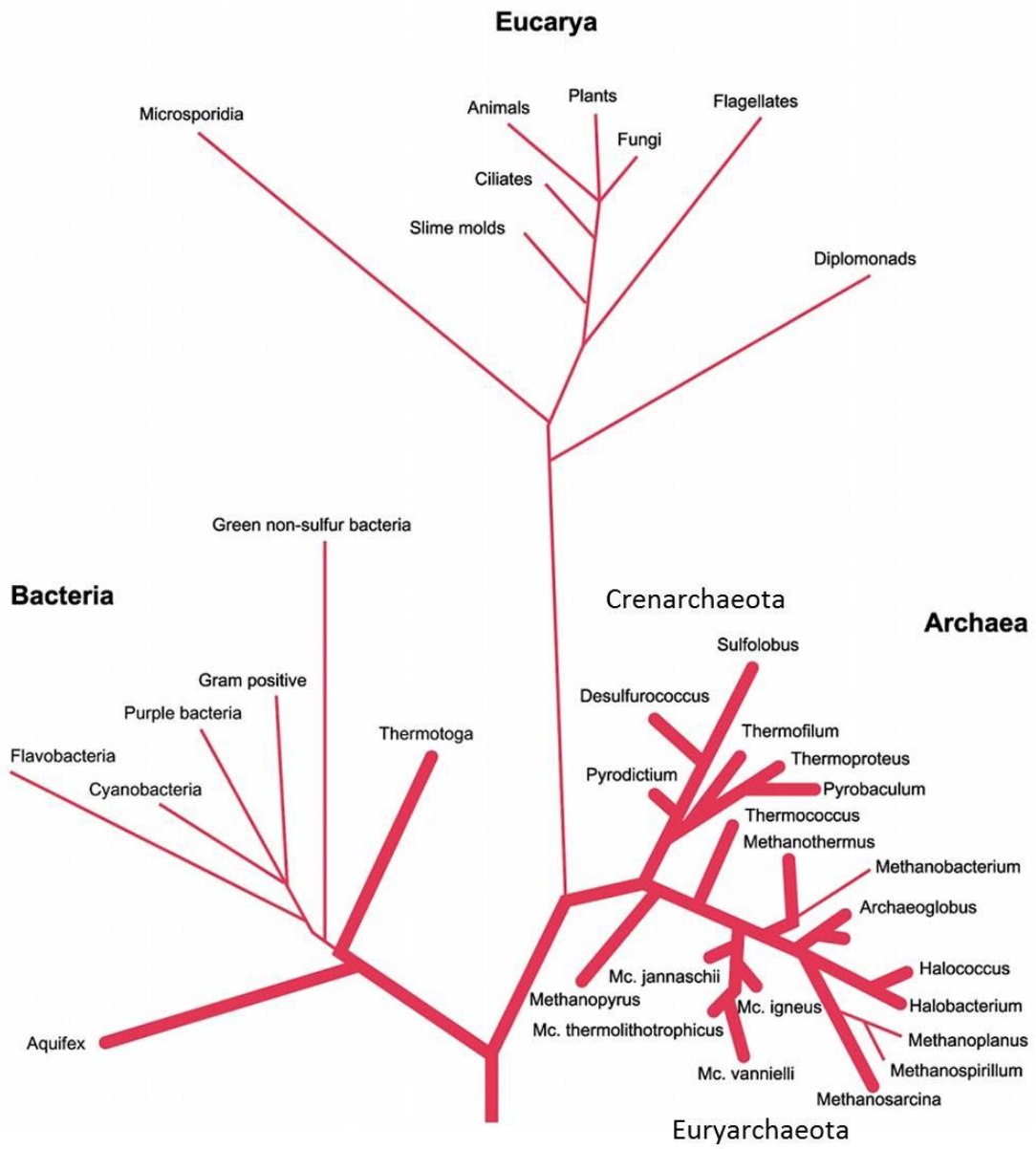

FIGURE 1.1. Phylogenetic tree of life based on 16S rRNA sequences, hyperthermophiles indicated in bold ((Xu & Glansdorff, 2002), modified from (Stetter, 1996)).

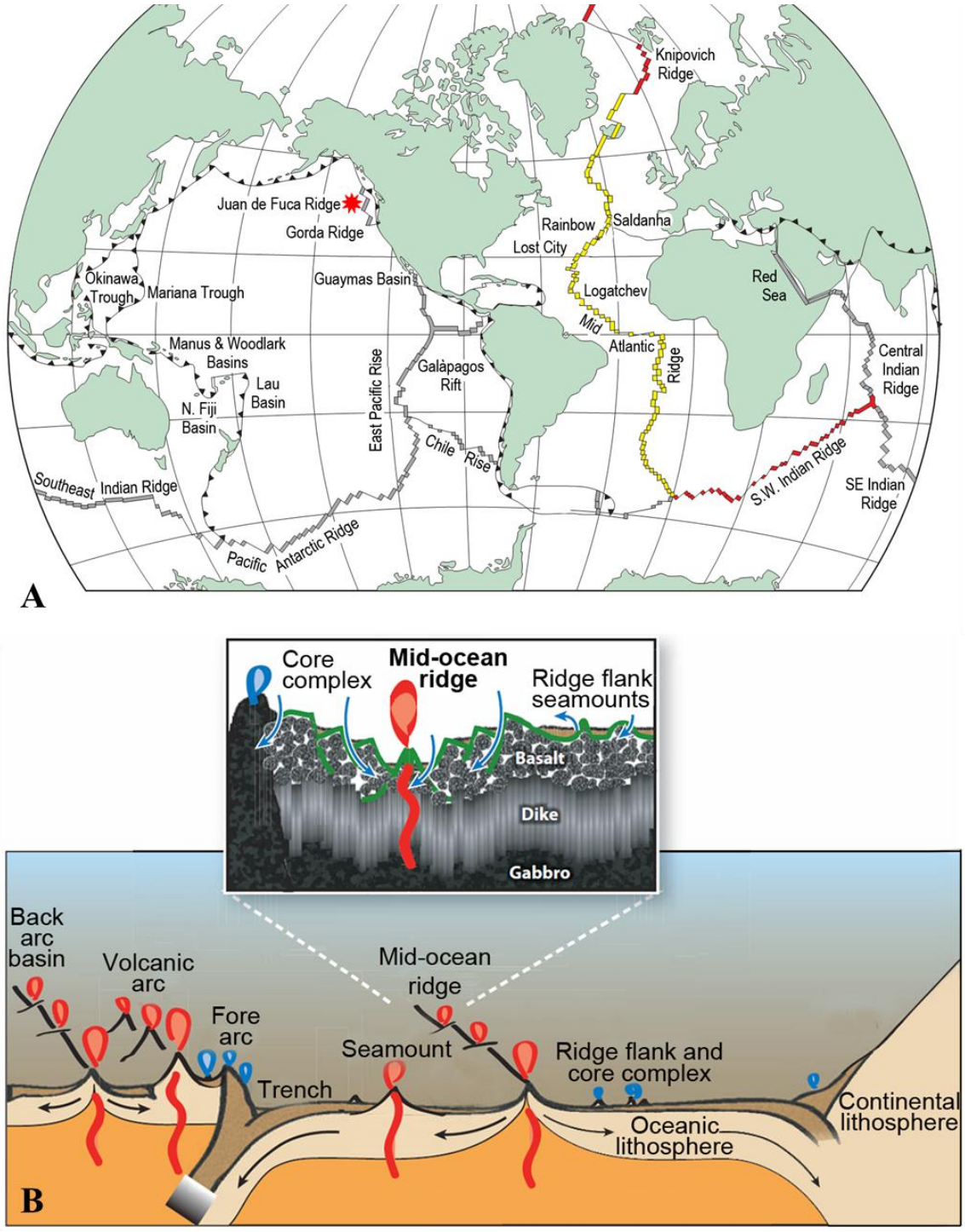

FIGURE 1.2. Schematics of hydrothermal vent formation. (A) Global mid-ocean ridge system, Juan de Fuca ridge indicated with star (German, 2004). (B) Tectonically driven geological forces that lead to hydrothermal venting (Schrenk *et al.*, 2010).

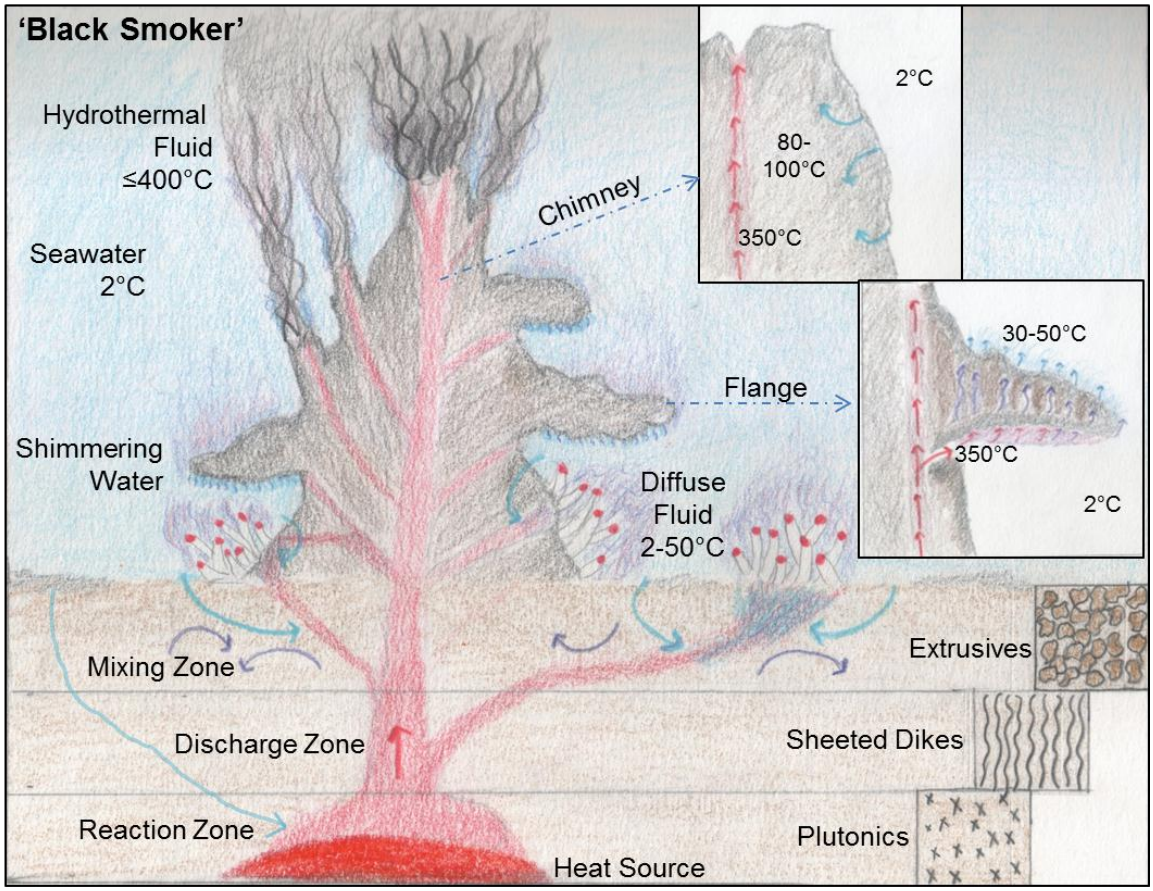

FIGURE 1.3. Fluid circulation within a hydrothermal vent system forming a sulfide edifice 'black smoker' chimney with flange structures.

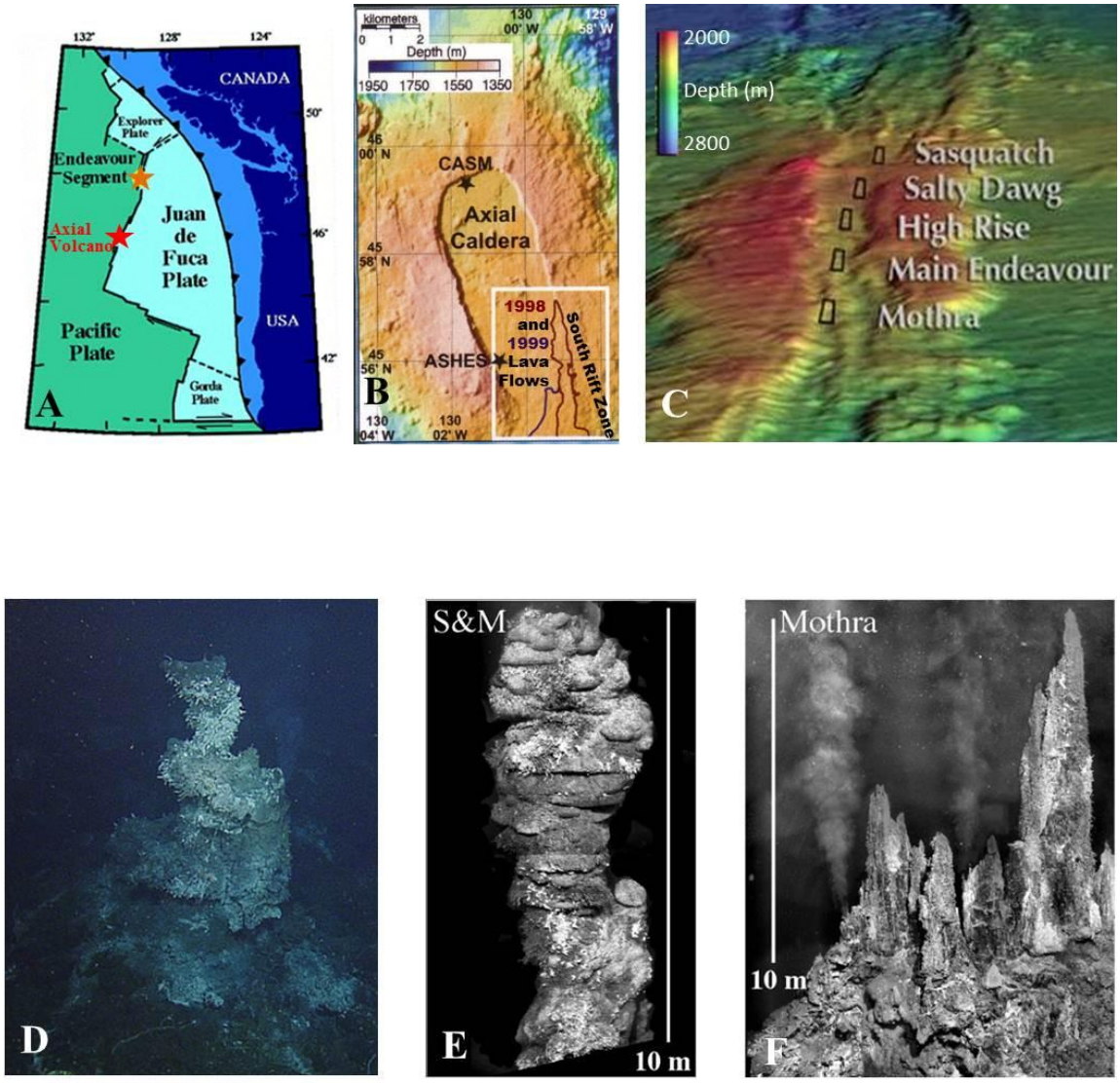

FIGURE 1.4. Deep-sea hydrothermal vent study sites. (A) Geographic location of the Endeavour Segment of the Juan de Fuca Ridge (orange star) and Axial Volcano (red star). (B) Bathymetry map of Axial, indicating the caldera, main venting regions and lava flows. (C) Bathymetry map of Endeavour, indicating the five known hydrothermal vent fields. (D) Small vent structure, typical of the ASHES field of Axial Volcano. (E) Smoke and Mirrors (S&M) vent structure, typical of the Main Endeavour Field. (F) Faulty Towers venting complex, typical of Mothra vent field of Endeavour. (Image credits: D. Kelley; (Butterfield *et al.*, 2004); Visions 2005; (Kelley *et al.*, 2002))

CHAPTER 2

ABUNDANCES AND CHARACTERISTICS OF HYPERTHERMOPHILIC AUTOTROPHIC IRON REDUCERS AND HETEROTROPHS IN HYDROTHERMAL VENT SYSTEMS ALONG THE ENDEAVOUR SEGMENT

2.1 Abstract

Relative abundances of hyperthermophilic autotrophic reducers of amorphous iron(III) oxide, methanogens and heterotrophs were measured in 16 deep-sea hydrothermal vent sulfide deposit samples (11 actively venting black smoker chimneys, 3 sulfide-hosted diffuse fluid samples, and two batches of 25 *Paralvinella sulfincola* polychaete worms) from the Endeavour Segment on the Juan de Fuca Ridge in the northeast Pacific Ocean. Hyperthermophilic autotrophic iron(III) oxide reducers were found in nearly all deep-sea hydrothermal vent samples collected. Their abundances based on most-probable-number estimates at 90°C were at least equal to and typically greater than those of hyperthermophilic heterotrophs in all but the polychaete sample where the heterotrophs significantly outnumbered the iron reducers. There was no growth of hyperthermophilic methanogens from any sample. Four hyperthermophilic iron(III) oxide reducing strains and 12 heterotrophic strains, respectively belonging to the *Pyrodictiaceae* and *Thermococcaceae* families were cultured and characterized. Results suggest that conditions favor the growth of heterotrophs towards the outer surface of massive sulfide deposits, near high-temperature polychaete worms; the hyperthermophile

populations then become increasingly autotrophic towards the interior, where autotrophic dissimilatory iron reducers are a common constituent.

2.2 Introduction

Hyperthermophiles (*i.e.*, organisms with optimal growth temperatures above 80°C (Stetter, 1996)) serve as models of microbial processes that occur at high *in situ* temperatures. They may also serve as tracers of the *in situ* chemical and physical conditions in marine systems since they are generally not found in surrounding seawater and their metabolisms are likely reflections of the chemistry and temperature of their environment (Holland *et al.*, 2004). Since the subseafloor is relatively inaccessible, there remains much to be learned about the distribution, abundances, and characteristics of the organisms inhabiting this subsurface environment. Hydrothermal venting serves as access points of this subsurface biosphere.

Deep-sea hydrothermal vents are common features along mid-ocean ridges where new seafloor is formed along oceanic plate spreading centers. Volcanically-derived gases and products from water-rock reactions in the basement rocks support chemolithoautotrophic-based microbial communities. Hydrothermal sulfide deposits form at deep-sea vents when upwelling high-temperature hydrothermal fluids mix with cold seawater resulting in precipitation of metal sulfides (Tivey *et al.*, 1995). Such systems not only entrain microbial material from depth, but the resultant sulfide deposits

host diverse microbial communities that thrive within their warm, porous interiors (Kormas *et al.*, 2006; McCliment *et al.*, 2006; Pagé *et al.*, 2008; Schrenk *et al.*, 2003).

One of the best studied deep-sea hydrothermal systems is the Endeavour Segment of the Juan de Fuca Ridge in the northeastern Pacific Ocean (47° 56.9'N, 129° 5.8'W, Figure 2.1), where massive sulfide deposits form (Delaney *et al.*, 1992; Glickson *et al.*, 2007; Robigou *et al.*, 1993). There are currently five major hydrothermal vent fields of the Endeavour Segment that are regularly spaced within a ~150 m deep axial valley: Sasquatch, Salty Dawg, High Rise, Main Endeavour, and Mothra (listed from North to South) (Figure 2.1). In a recovered actively venting structure (Delaney *et al.*, 2001), approximately 65% of the total prokaryotic population in its interior was archaeal, based on fluorescent *in situ* hybridization (FISH) analysis, with the majority of these being unknown Crenarchaeota (Schrenk *et al.*, 2003). The proportions of *Methanocaldococcaceae* and *Thermococcaceae* (both hyperthermophilic families within the Euryarchaeota) were very low (≤ 4%) throughout the entire structure. A novel hyperthermophilic member of the Crenarchaeotal order *Desulfurococcales* was isolated from the same deposit that is an obligate autotrophic reducer of amorphous iron(III) oxide with an optimum growth temperature of 106°C (Kashefi & Lovley, 2003). A recent study analyzed a recovered sulfide from a nearby structure and found that *Desulfurococcales* predominated, comprising nearly 50% of archaeal sequence clones, while methanogens were below the detection limit (Zhou *et al.*, 2009). There are few reports of hyperthermophilic reducers of amorphous iron(III) oxide isolated from deep-sea hydrothermal environments (Kashefi *et al.*, 2002; Kashefi *et al.*, 2008); therefore, determining their potential relevance at Endeavour was a goal of this study.

While molecular assessments of microbial diversity in hydrothermal sulfide deposits are common and indeed necessary (see Schrenk *et al.,* 2008 for a review), there are relatively few reports on the metabolic diversity and microbial abundances in submarine vents using select culturing of indicator organisms. To address these issues, the purposes of this study were to determine: (1) whether hyperthermophilic autotrophic dissimilatory iron reducers are common in metal sulfide chimneys at the Endeavour Segment, (2) whether these organisms are significantly higher in abundance than hyperthermophilic methanogens, whose growth conditions differ significantly from those of iron reducers in mesophilic environments (Lovley & Goodwin, 1988; Lovley *et al.*, 1994), and (3) how their abundances vary with those of hyperthermophilic heterotrophs throughout a sulfide deposit. This study also examines some of the growth characteristics of hyperthermophilic autotrophic iron(III) oxide reducers and obligate heterotrophs. In particular, the heterotrophs were screened for their ability to grow on maltose and cellobiose for potential H_2 biofuel applications.

2.3 Materials and Methods

2.3.1 Sample Collection

Samples were collected from the Endeavour Segment of the Juan de Fuca Ridge (Figure 2.1) in May-June 2004, August-September 2006, and August-September 2008 using the deep-sea research submarine *Alvin*. The sample sites were the Mothra, Main and High Rise hydrothermal vent fields, which host steep-sided, porous sulfide mineral

deposits that can rise > 30 m above the surrounding seafloor. The structures were emitting hydrothermal fluids up to 363°C (Table 2.1). In 2004, samples used for this study were recovered from six actively-venting black smoker chimneys from the Mothra and Main fields (Table 2.1). In 2006, another seven actively-venting chimneys were sampled from the Mothra, Main and High Rise fields (Figure 2.2A-B). Three sulfide-hosted diffuse fluid samples (5-140°C) were collected in 2006 with 750-ml titanium syringes from the Mothra and Main fields (Figure 2.2C). Two batches of 25 *Paralvinella sulfincola* polychaete worms were collected from the outer surfaces of active sulfide mounds at the Mothra (2006) and Main (2008) fields (Figure 2.2D).

Once samples were onboard the research vessel *Atlantis*, 12 to 24 g of the soft, porous marcasite-wurtzite-sphalerite-rich material from the interior of the sulfide chimneys were added to 50 ml of sterile, anoxic artificial seawater. This solution was composed of the salts in DSMZ Medium 141 (www.dsmz.de/media/med141.htm). After adding the sample, the bottle was sealed with a butyl stopper, flushed with N_2:CO_2 (70:30) and reduced with 0.025% (wt vol^{-1}) each of cysteine-HCl•H_2O and Na_2S•$9H_2O$. Twenty-five *P. sulfincola* worms were separated from other macrofauna and sulfide material collected by *Alvin* as part of a larger biology sample, cut in half, and added to DSMZ Medium 141 salts as described above. Low-temperature diffuse hydrothermal fluids (50 ml) collected using titanium syringes were transferred immediately onboard the ship into a sealed serum bottle that had been flushed with N_2:CO_2 (80:20) and reduced with the cysteine-sulfide solution as before.

2.3.2 Growth Media

The medium for dissimilatory iron reducers was based on Kashefi Marine Medium (Kashefi *et al.*, 2002) with a 2 atm H_2:CO_2 (80:20) headspace as the electron donor and carbon source, 100 mmol liter^{-1} Fe(III) oxide as the terminal electron acceptor, and 0.5 mM cysteine-HCl•H_2O as the reducing agent. $FeCl_2$ (1.3 mM) was added to the medium immediately prior to inoculation. The heterotroph and methanogen media were made in DSMZ Medium 141 salts. The heterotroph medium contained 0.5% (wt vol^{-1}) each of yeast extract and peptides, 0.1% (wt vol^{-1}) elemental sulfur as the terminal electron acceptor, and 0.025% (wt vol^{-1}) each of cysteine-HCl•H_2O and Na_2S•$9H_2O$. The methanogen medium had 2 atm H_2:CO_2 (80:20) as a headspace, 0.02% yeast extract to provide trace nutrients to initiate growth, and 0.025% (wt vol^{-1}) each of cysteine-HCl•H_2O and Na_2S•$9H_2O$ as the reducing agents. All media were pH balanced at 6.80 ± 0.05.

2.3.3 Enrichments and Most-Probable-Number (MPN) Estimates

Efforts in 2004 to culture hyperthermophilic archaea at 95°C from six black smoker chimneys on eight types of media (autotroph: NO_3^-, S°, Fe(III) reduction, and methanogen media; heterotroph: NO_3^-, S°, SO_4^{2-}, and Fe(III) reduction media) resulted solely in the growth of autotrophic dissimilatory iron(III) oxide reducers (4 samples) and obligate heterotrophs (5 samples). Therefore, further hyperthermophile enrichments were made in the media described in the above section and incubated at 95°C for up to 7 days.

To determine the abundances of these organisms and those of hydrogenotrophic methanogens, three-tube MPN estimates were conducted using 12 samples (Table 2.1).

Three-tube MPN analyses (Holden *et al.*, 1998) were performed by adding 3.33 ml, 0.33 ml, and 0.03 ml of each sample in triplicate to the heterotroph, methanogen, and iron(III) oxide reducer media. After inoculation, the tubes were incubated at 90°C for up to 7 days. Growth in the tubes was confirmed for all three types of media using phase-contrast light microscopy. Growth of dissimilatory iron reducers was determined spectrophotometrically by testing for the production of iron(II) using the Ferrozine method (Lovley & Phillips, 1986). Growth of methanogens and H_2-producing heterotrophs was determined by analyzing the headspace with gas chromatography for CH_4 and H_2, respectively.

Cell concentrations in black smoker chimney samples were normalized to the dry weight of sediment contained within 1 ml of sample slurry drawn by a syringe. Attempts to determine the total cell concentrations by using acridine orange staining and epifluorescence microscopy were unsuccessful due to cell concentrations that were below the detection limit ($< 10^4$ cells ml^{-1}).

2.3.4 Hyperthermophile Culturing and Characterizations

The positive enrichments from 2004 and the MPN samples from 2006 were used as inocula to isolate pure cultures of hyperthermophiles. Hyperthermophilic strains were purified through three sets of 10-fold dilution-to-extinction incubations at either 90°C or 95°C depending on the original incubation temperature.

The iron(III) oxide reducer strains were tested for their ability to grow with either 0.05% (wt vol^{-1}) sodium acetate or 0.1% (wt vol^{-1}) casein hydrolysate and 0.05% (wt vol^{-1}) yeast extract in the medium with either 2 atm H_2:CO_2 (80:20) or 2 atm Ar in the

headspace. Each of these contained 100 mmol liter^{-1} iron(III) oxide as the terminal electron acceptor. They were also tested for their ability to grow autotrophically with either 10 mM $NaNO_3$, 10 mM Na_2SO_4, 8 mM $Na_2S_2O_3$, 0.1% elemental sulfur (wt vol^{-1}), or 1-2% (vol vol^{-1}) O_2 as the terminal electron acceptor.

The heterotroph strains were tested for their ability to grow on 0.5% (wt vol^{-1}) casein hydrolysate, 0.5% (wt vol^{-1}) maltose, or 0.5% (wt vol^{-1}) cellobiose. Each of these heterotrophic media also contained 0.05% (wt vol^{-1}) yeast extract, which could not sustain growth alone. Growth on each of these media was tested with and without the addition of 0.1% (wt vol^{-1}) elemental sulfur.

Growth was only considered to be positive after three successive transfers on the medium. Cell concentrations of iron(III) oxide reducers were determined by dissolving the iron in the sample using an oxalate solution (28 g l^{-1} ammonium oxalate and 15 g l^{-1} oxalic acid), filtering the cells onto a 0.2-μm-pore-size polycarbonate filter prestained with Irgalan black, staining with acridine orange (Daley & Hobbie, 1975), and counting using epifluorescence microscopy. Heterotroph cell concentrations were determined using a Petroff-Hausser counting chamber and phase-contrast light microscopy.

DNA from each isolated strain was extracted using either a genomic DNA purification kit (Wizard Genomic DNA purification kit, Promega) with the addition of 0.1 mg proteinase K or a phenol-chloroform DNA extraction. The amount and quality of the DNA samples were determined using a Nano-drop spectrophotometer. The universal archaeal 16S rRNA primers 21f (5′-TTCCGGTTGATCCYGCCGGA-3′) and 958r (5′-YCCGGCGTTGAMTCCAATT-3′) were used for DNA amplification using PCR. The PCR products were purified and concentrated using a PCR purification kit (QIAquick,

Qiagen) and sequenced in both directions using 21f and 958r as the primers. These sequences were then compared with other known nucleotide sequences in the NCBI database using a BLAST analysis (Altschul *et al.*, 1990). Similar sequences were aligned using BioEdit (Hall, 1999) and ClustalW (Larkin *et al.*, 2007) alignment software and phylogenetic trees were created using Mega4 (Tamura *et al.*, 2007) software.

2.3.5 Organisms Used

Pyrodictium abyssi DSM6158, *Pyrolobus fumarii* DSM11204, *Pyrococcus furiosus* DSM3638, *Thermococcus* sp. strain ES1 and *Desulfurococcus* sp. strain SY were used for comparison for this study. *P. abyssi*, *P. fumarii* and *P. furiosus* were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *Thermococcus* strain ES1 and *Desulfurococcus* strain SY were kindly provided by John Baross (University of Washington) and Holger Jannasch (Woods Hole Oceanographic Institution), respectively.

2.4 Results

2.4.1 MPN Estimates

Up to 99 iron(III) oxide reducer cells per gram dry weight of sulfide material were measured in each of the seven black smoker chimneys examined (Table 2.1). In six of these, the estimated number of autotrophic iron reducers was higher than those of the heterotrophs. Up to 33,000 iron(III) oxide reducer cells per liter were measured in

diffuse fluids emitted from the interior of the sulfide deposits. Similarly, in these fluids the estimated number of iron(III) oxide reducers was higher than those of the heterotrophs. For the worm samples, all nine heterotroph MPN tubes were turbid following incubation, indicating that the number of heterotrophs exceeded the estimated range of the MPN test, 144 cells per worm. The 2008 worm sample was further diluted to achieve a more accurate estimate of up to 2,580 heterotroph cells per worm. In contrast to the other sample types, the estimates of iron(III) oxide reducers within the worm samples were significantly lower than those of the heterotrophs. Similar to results from 2004, hydrogenotrophic methanogens were not detected in any of the samples.

2.4.2 Characteristics of the Iron(III) Oxide Reducers

Four strains of autotrophic iron reducers were chosen for further characterization. Phylogenetic analysis identified two of these strains (Su06 and HH04) as members of the archaeal genus *Pyrodictium* and the other two strains (Ro04 and BW06-2) as members of the closely related genus *Hyperthermus* (Figure 2.3). HH04 and BW06-2 grew in the presence of acetate with an H_2 and CO_2 headspace, although neither their growth rates nor their maximum cell concentrations increased significantly relative to growth on H_2 and CO_2 alone (Table 2.2). Growth of the other two strains was inhibited by acetate. All four strains grew in the presence of peptides with H_2 and CO_2 in the headspace, but their growth rates and maximum cell concentrations also were not different from those cultures grown autotrophically. Only *Hyperthermus* strain BW06-2 grew on either acetate or peptides with argon in the headspace. *Hyperthermus* strains Ro04 and BW06-2 grew when nitrate was the terminal electron acceptor (Table 2.3). For BW06-2, the maximum

cell concentration increased to more than 10^8 cells ml^{-1} when grown on nitrate. All four strains grown on iron(III) oxide only reached 3×10^7 cells ml^{-1}. BW06-2 also grew using sulfate as the terminal electron acceptor, albeit to a lower cell concentration than when grown on iron(III) oxide. Neither *Pyrodictium* strain (HH04 and Su06) grew on any of the other potential electron acceptors tested, and Ro04 and BW06-2 did not grow with thiosulfate, elemental sulfur, or low O_2. *P. abyssi* and *P. fumarii* also grew using iron(III) oxide as a terminal electron acceptor up to cell concentrations that were comparable to those observed in this study.

2.4.3 Characteristics of the Heterotrophs

Twelve strains of heterotrophs were chosen for further characterization. Phylogenetic analyses identified six of these strains as members of the archaeal genus *Pyrococcus* and six strains as members of the closely related genus *Thermococcus* (Figure 2.3). None of them grew autotrophically with H_2 and CO_2 as the sole energy and carbon sources. All of the strains tested grew on peptides, maltose, and cellobiose when elemental sulfur was present (Table 2.4). Nearly all of these strains grew to cell concentrations greater than 10^8 cells ml^{-1}. Furthermore, even when grown on elemental sulfur, all but one of the strains produced H_2 and H_2S as end products. Three of the strains grew on peptides and maltose without sulfur present, and two strains grew on cellobiose without sulfur. H_2 was produced by these cultures. Similarly, *Pyrococcus furiosus* and *Thermococcus* strain ES1 grew on peptides, maltose, and cellobiose with and without sulfur, and *Desulfurococcus* strain SY grew in all of these media except peptides without sulfur.

2.5 Discussion

Within submarine hydrothermal systems there is a critical need to determine the relative abundances, distributions, and metabolic characteristics of so-called indicator organisms. Such organisms may provide insight into the chemical conditions present in environments that are not readily accessible to study, such as the interior walls of deep-sea vents or the subsurface biosphere. Investigation of these indicator organisms may also provide insights into the characteristics of microbes present even when they themselves are not necessarily the most abundant or biogeochemically important organisms (Holland *et al.*, 2004). Hydrothermal vent systems may be significantly diverse, as indicated by a study that reported finding approximately 37,000 bacterial and 2,600 archaeal operational taxonomic units (OTUs) in two low-temperature deep-sea hydrothermal fluid samples collected from Axial Volcano in the northeastern Pacific Ocean (Huber *et al.*, 2007). Studies that examined subsections of recovered sulfide deposits found that microbial abundance and diversity decreases towards the hot interior (Harmsen *et al.*, 1997; Takai *et al.*, 2001). Some of the more fundamental questions about vents concern the distributions of autotrophs versus heterotrophs and the distribution of various autotrophs with differing growth requirements. Given these unknowns, hyperthermophiles make ideal indicator organisms since they are absent in background seawater that contaminates many hydrothermal fluid and rock samples and they generally reflect the chemistry of their environment.

The MPN results presented in this study indicate that hyperthermophilic heterotrophs are most abundant near the outer surface of black smoker chimneys in close

proximity to vent metazoans and that there is a transition to more autotrophic hyperthermophile communities towards the interior of the chimney walls (Table 2.1). The polychaete worm *P. sulfincola* was chosen as a source of hyperthermophiles in this study because it burrows into black smoker chimneys, can live at temperatures that exceed other metazoans at deep-sea vents (up to 55°C) (Girguis & Lee, 2006), and they have previously been shown to be a source of hyperthermophilic heterotrophs (Holden *et al.*, 2001; Pledger & Baross, 1989).

Based on culturing, iron(III) oxide reducers appear to be more abundant than methanogens within the autotroph communities. Hyperthermophilic methanogens have been previously found in 90°C MPN analyses of low-temperature basalt-hosted hydrothermal fluids from the Endeavour site (Holden *et al.*, 1998) and in molecular and FISH analyses of Endeavour sulfide deposits (Schrenk *et al.*, 2003). We cannot exclude the possibility of a culturing bias or other potentially limiting factors such as O_2 exposure during sample recovery or the possibility that the organisms are viable but not culturable. However, other possible explanations for the absence of hyperthermophilic methanogens include environmental conditions that limit their growth such as H_2 limitation and reduction potentials that are more positive than the minimum needed for growth.

A comparison of H_2 concentrations in hydrothermal fluids from Endeavour (Lilley *et al.*, 1993; Lilley *et al.*, 2003) with thermodynamic modeling of hydrogenotrophic methanogenesis (Hoehler, 2004) suggests that the hydrogen activity of the fluids may be below the minimum need for growth. In contrast, the minimum H_2 activity needed for growth of iron(III) oxide reducers should be significantly less than that of methanogenesis due to the higher free energy available from the reduction of

iron(III) oxide (Amend & Shock, 2001). The transition from highly reducing to oxidizing conditions in the interior of sulfide deposits is predicted to be sharp with decreasing temperature (McCollom & Shock, 1997; Tivey, 2004). Since these systems are highly dynamic, highly oxidizing conditions may also occur locally at higher temperatures (*e.g.*, 90°C) within the chimney walls if there is significant influx of seawater into the deposit (Tivey, 2004).

Evidence that significant seawater entrainment does occur in these systems is provided by detailed mapping and mineral analyses of sulfide chimneys from the Finn and Roane sulfide structures in Mothra vent field, which at the time of sampling were venting 304°C and 278°C fluid, respectively. Within Finn, high-angle fractures provide channels for focused flow of seawater directly into the main high-temperature through-going fluid channel and into the interior walls as evident by the presence of a chalcopyrite encased anhydrite "horn" within the main conduit and pockets of anhydrite in the interior walls. Anhydrite ($CaSO_4$) forms during heating of seawater above 150°C (Bischoff & Seyfried, 1978). Within Roane, anhydrite is extremely rare, however, up to 20 modal percent gordaite ($NaZn_4(SO_4)Cl(OH)_6 \bullet 6H_2O$) was present as a void-filling mineral within the most interior portions of the chimney (Kristall *et al.*, 2006). Petrographic relationships indicate that this mineral precipitated directly from mixed hydrothermal fluid and seawater during the late stage growth evolution of this chimney.

Conditions such as those documented in Finn and Roane would likely favor the growth of hyperthermophilic iron(III) oxide reducers, which generally prefer relatively more oxidizing conditions (Feinberg *et al.*, 2008), over hydrogenotrophic methanogens that require reduction potentials below -330 mV (Sowers, 1995). Hyperthermophilic

methanogens are likely to be present in highly reduced pockets, perhaps in close proximity to hyperthermophilic heterotrophs that can provide H_2 and reductant (*e.g.*, H_2S) for growth.

Geoglobus ahangari (Kashefi *et al.*, 2002) and a member of the *Pyrodictiaceae* (strain 121 (Kashefi & Lovley, 2003)) are the only two obligate hyperthermophilic iron reducer strains from deep-sea hydrothermal vent sites that have been characterized. They have optimal growth temperatures of 88 and 106°C, respectively. In this study, we isolated four new strains of hyperthermophilic autotrophic iron(III) oxide reducers, all of which belong to the *Pyrodictiaceae* family. The two *Pyrodictium* strains are obligate iron(III) oxide reducers while the two *Hyperthermus* strains also use nitrate as a terminal electron acceptor (Table 2.3). For strain BW06-2, growth rates and maximum cell concentrations were higher on nitrate relative to iron(III) oxide while growth rates and maximum cell concentrations decreased for strain Ro04 when grown on nitrate. Two other previously cultured members of the *Pyrodictiaceae* (*P. abyssi* and *P. fumarii*) that were likewise isolated from deep-sea hydrothermal sulfide deposits (Blöchl *et al.*, 1997; Pley *et al.*, 1991) also grew on iron(III) oxide and produced magnetic iron(II) as an end product suggesting that the ability to grow on insoluble iron oxide is widespread among this group of hyperthermophiles.

All 12 obligate heterotroph strains grew on peptides, maltose, and cellobiose when elemental sulfur was present (Table 2.4). Maltose and cellobiose are glucose disaccharides with α-1,4 and β-1,4 linkages, respectively, and are the products of starch and cellulose breakdown. Three strains grew on these without the addition of sulfur with concomitant production of H_2. Therefore, they are well adapted to growth on several

organic compounds that likely come from vent animal secretions and the biofilms of other microorganisms. A potential application of these and similar strains is for H_2 biofuel production in a consolidated bioprocessing reactor using starch or cellulose as a feedstock. *Pyrococcus furiosus* was shown here and elsewhere (Chou *et al.*, 2007) to use maltose and cellobiose for growth and H_2 production, and also encodes a functional β-1,4-endoglucanase for cellulose degradation in its genome (Bauer *et al.*, 1999). A later study reported that microbial growth rates on cellulose increase with increasing temperature but did not examine growth above 74°C (Lynd *et al.*, 2002). Therefore, the prospects of using some of the hyperthermophilic heterotrophs analyzed in this study for industrial H_2 biofuel production using agricultural feedstocks are promising.

In conclusion, hyperthermophilic autotrophic iron(III) oxide reducers were found in each sample from the Endeavour Segment hydrothermal vent fields. They were the only hyperthermophilic autotrophs cultured from these samples and were generally more abundant than obligate heterotrophs in all but the animal-associated sample, suggesting that they are significant constituents of the hyperthermophile population in Endeavour sulfide deposits. Under these experimental conditions hyperthermophilic methanogens were absent, which is interesting since they have been found previously at Endeavour (Holden *et al.*, 1998; Schrenk *et al.*, 2003). Possible explanations for the absence of hyperthermophilic methanogens include environmental conditions that limit their growth such as low H_2 concentrations and/or low reduction potentials. Future studies will model the constraints on the growth of hyperthermophilic iron reducers and methanogens and the rates of metabolite production. The long-term goal is to determine the biogeochemical impact of these and other microorganisms on hydrothermal vent systems

and how this may translate into a better understanding of life deeper within the Earth's crust.

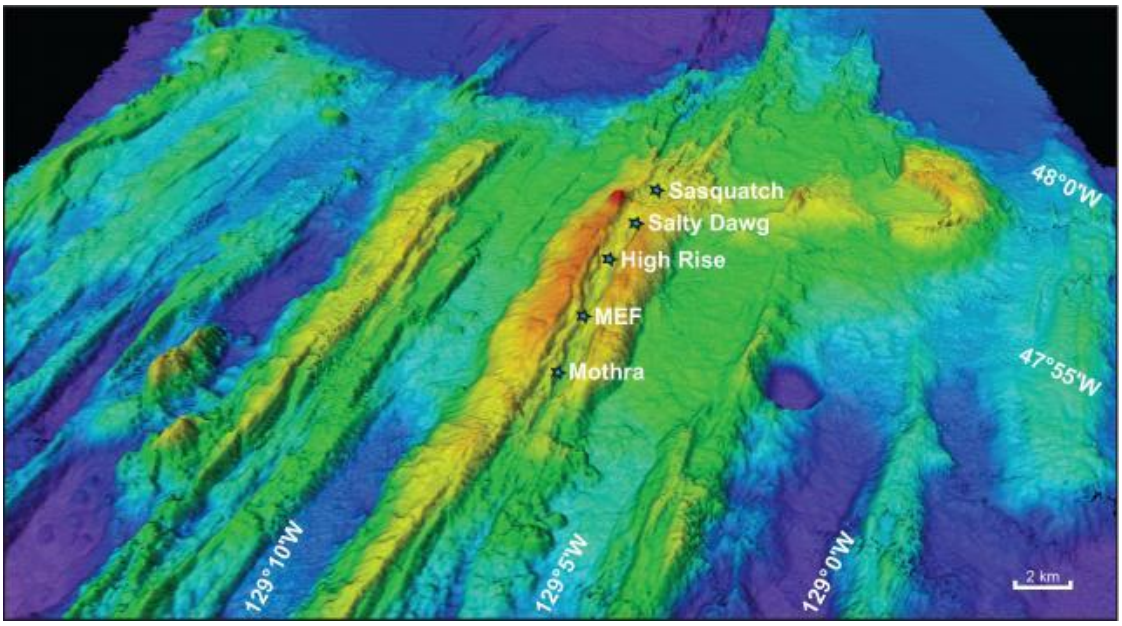

FIGURE 2.1. Shaded relief bathymetry of the Endeavour Segment in the northeastern Pacific Ocean. Hydrothermal vent field locations are indicated by stars. The color ranges from 1,750 m (red) to 3,000 m (blue) (Glickson *et al.*, 2007).

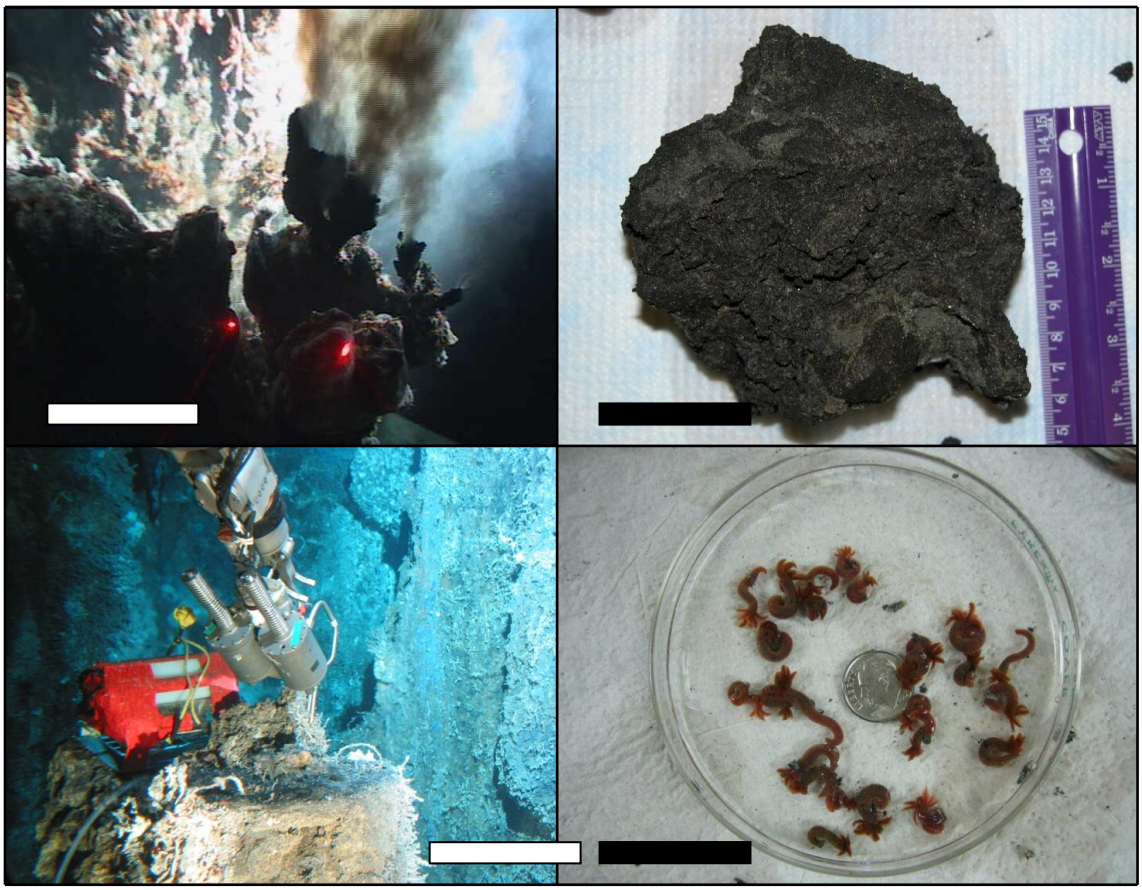

FIGURE 2.2. Samples collected from the Endeavour Segment. (A) A black smoker chimney on the Boardwalk sulfide deposit (scale bar = 10 cm). (B) Wurtzite-sphalerite rich material from the Hulk sulfide deposit used for culturing (scale bar = 4 cm). (C) Low-temperature fluid collected with titanium syringes from the Roane sulfide deposit (scale bar = 0.5 m). (D) *Paralvinella sulfincola* polychaete worms collected from the Salut sulfide deposit (scale bar = 3 cm).

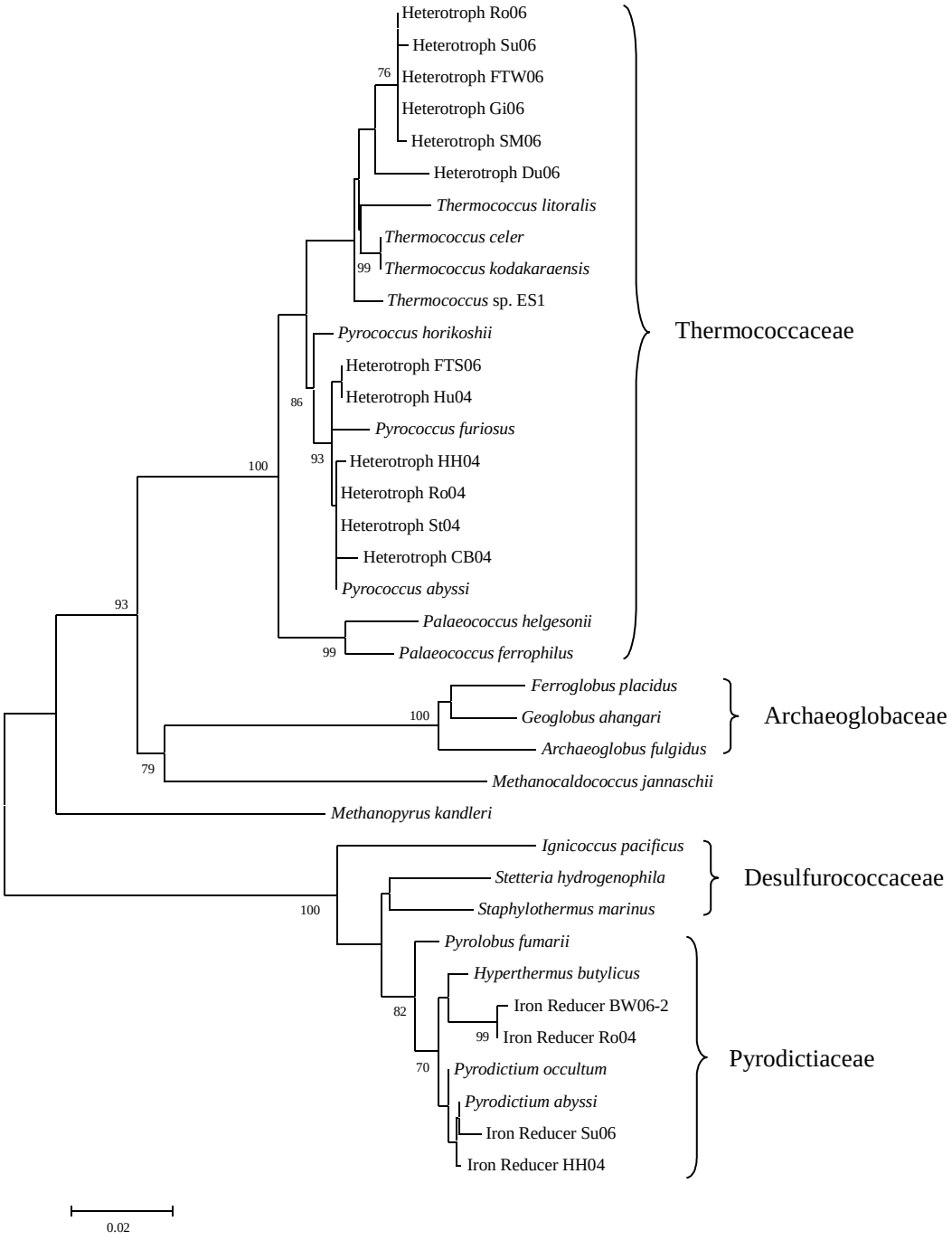

FIGURE 2.3. 16S rRNA phylogenetic tree showing the taxonomic locations of the cultured hyperthermophilic autotrophic iron reducers and obligate heterotrophs relative to other marine hyperthermophilic archaea.

TABLE 2.1. Abundances of hyperthermophilic autotrophic iron(III) oxide reducers, methanogens and heterotrophic sulfur reducers grown at 90°C[a]

Sample ID	Sample description	Most-probable-number estimates		
		Autotrophic Fe(III) reducers	Hydrogenotrophic methanogens	Heterotrophic sulfur reducers
Gi06	Sulfide material from Giraffe vent	91 g^{-1}	ND[b]	91g^{-1}
Su06	Sulfide material from Sully vent, 349°C fluids[c]	85 g^{-1}	ND	15 g^{-1}
Du06	Sulfide material from Dudley vent, 327°C fluids	22 g^{-1}	ND	7 g^{-1}
Hu06	Sulfide material from Hulk vent, 316°C fluids	41 g^{-1}	ND	ND
BW06-1	Sulfide material from Boardwalk vent, 356°C fluids	78 g^{-1}	ND	2 g^{-1}
BW06-2	Sulfide material from Boardwalk vent, 356°C fluids	91 g^{-1}	ND	41 g^{-1}
FTS06	Sulfide material from Faulty Towers vent	99 g^{-1}	ND	14 g^{-1}
SM06	Diffuse fluids (140°C) from S&M vent	13,800 L^{-1}	ND	840 L^{-1}
Ro06	Diffuse fluids (5°C) from Roane vent	840 L^{-1}	ND	270 L^{-1}
Ph06	Diffuse fluids (48°C) from Phang vent	33,000 L^{-1}	ND	ND
FTW06	25 *P. sulfincola* worms from Faulty Towers vent	13 $worm^{-1}$	ND	>144 $worm^{-1}$[d]
Sa08-2	25 *P. sulfincola* worms from Salut vent	1 $worm^{-1}$	ND	2,580 $worm^{-1}$[d]

[a]Estimates are per gram of dry weight sulfide material, per liter of fluid collected, and per *P. sulfincola* worm.
[b]ND, not detectable.
[c]Fluid temperatures are for hydrothermal end-member fluids flowing out of the sulfide deposits at the time of collection.
[d]For FTW06, all 9 MPN tubes showed growth indicating that the sample cell concentration exceeded the range of the MPN test. For Sa08-2, the sample was diluted 1:1000 prior to the MPN test. The Sa08 MPNs were run in 2008 at 85°C instead of 90°C.

TABLE 2.2. Growth of the hyperthermophilic iron(III) oxide reducers on various carbon sources and electron donors[a]

Isolate	Incubation temp.	Carbon and electron source				
		$H_2 + CO_2$	acetate/$H_2 + CO_2$	acetate/argon	peptide/$H_2 + CO_2$	peptide/argon
Pyrodictium Su06	90°C	++	-	-	++	-
Pyrodictium HH04	95°C	++	++	-	++	-
Hyperthermus Ro04	95°C	++	-	-	++	-
Hyperthermus BW06-2	90°C	++	++	++	++	++

[a]Table is scored based on maximum cell concentration measured using the following scale: +++, at least 10^8 cells ml^{-1}; ++, at least 3×10^7 cells ml^{-1}; +, at least 10^7 cells ml^{-1}; and -, no growth.

TABLE 2.3. Autotrophic growth of the hyperthermophilic iron(III) oxide reducers on other terminal electron acceptor[a]

Isolate	Incubation temp.	Terminal electron acceptor					
		Fe(III) oxide	KNO_3	Na_2SO_4	$Na_2S_2O_3$	S°	1-2% O_2
Pyrodictium Su06	90°C	++	-	-	-	-	-
Pyrodictium HH04	95°C	++	-	-	-	-	-
Hyperthermus Ro04	95°C	++	++	-	-	-	-
Hyperthermus BW06-2	90°C	++	+++	+	-	-	-

[a]Table is scored based on maximum cell concentration measured using the following scale: +++, at least 10^8 cells ml^{-1}; ++, at least 3×10^7 cells ml^{-1}; +, at least 10^7 cells ml^{-1}; and -, no growth.

TABLE 2.4. Growth of the hyperthermophilic heterotrophs on various carbon sources with and without the addition of elemental sulfur[a]

Isolate	Incubation temp.	Carbon source ± elemental sulfur					
		peptide + S°	maltose + S°	cellobiose + S°	peptide	maltose	cellobiose
Pyrococcus HH04	95°C	+++	+++	+++	-	-	-
Pyrococcus Ro04	95°C	+++	+++	+++	-	-	-
Pyrococcus St04	95°C	+++	+++	+++	-	-	-
Pyrococcus CB04	95°C	+++	+++	+++	-	-	-
Pyrococcus Hu04	95°C	+++	++	++	-	-	-
Pyrococcus FTS06	85°C	+++	+++	+++	-	-	-
Thermococcus Su06	85°C	+++	++	+++	+	+	+
Thermococcus Du06	85°C	+++	+++	+++	+	+++	-
Thermococcus SM06	85°C	+++	+++	+++	+	+	+
Thermococcus Ro06	85°C	+++	+++	+++	-	-	-
Thermococcus FTW06	85°C	+++	+++	+++	-	-	-
Pyrococcus furiosus	95°C	+++	+++	+++	+	+++	+++
Thermococcus ES1	85°C	+++	+++	+++	++	+++	++
Desulfurococcus SY	85°C	+++	+++	+++	-	+	+

[a]Table is scored based on maximum cell concentration measured using the following scale: +++, at least 10^8 cells ml^{-1}; ++, at least 3×10^7 cells ml^{-1}; +, at least 10^7 cells ml^{-1}; and -, no growth.

CHAPTER 3

H_2-LIMITED GROWTH OF HYPERTHERMOPHILIC METHANOGENS IN DEEP-SEA HYDROTHERMAL VENTS

3.1 Abstract

The purpose of this study was to determine whether an environmental parameter, namely hydrogen concentration, can impact hyperthermophilic methanogen distribution patterns. The growth kinetics and minimum H_2 concentration required for hyperthermophilic methanogenesis were defined and correlated with field microbiology and fluid geochemistry results from two hydrothermal vent sites in the northeastern Pacific Ocean and other published results. Growth of three *Methanocaldococcus* sp. at 70°C and 82°C in a gas flow-controlled bioreactor ceased below 17-23 μM H_2. This lower limit is more than 1000-fold higher than those reported for mesophilic methanogens indicating high maintenance energy costs of hyperthermophilic methanogenesis. In field studies, anomalously high CH_4 concentrations and thermophilic methanogens were observed in low-temperature hydrothermal fluids only where H_2 concentrations were above this predicted threshold. *In situ*, hyperthermophilic heterotrophs outnumber methanogens, and co-culture experiments suggest hyperthermophilic methanogens rely on heterotrophic H_2 when H_2 limited. This study establishes a threshold for hyperthermophilic methanogen growth based on H_2 and explains in part the global microbial diversity patterns observed in mid-ocean ridge basalt

and volcanic arc hydrothermal environments relative to ultramafic and post-eruptive vent sites.

3.2 Introduction

There are estimates that as much as a third of the total biomass of the planet exists within the seafloor (Whitman *et al.*, 1998), and a major fraction of this biomass may be archaea (Lipp *et al.*, 2008). These estimates have led to a renewed interest in quantitatively modeling the habitability, CO_2 fixation, and biogeochemical cycling within the seafloor. Our understanding of CO_2 fixation in the deep-sea and the subseafloor and its impact on marine biogeochemical cycling is in its infancy, but it is known that hydrothermal vents are among the most productive regions within these zones. Methanogens of the order *Methanococcales* are among the more commonly found autotrophs in vent environments (Higashi *et al.*, 2004; Huber *et al.*, 2002b; Nakagawa *et al.*, 2005; Nercessian *et al.*, 2005; Perner *et al.*, 2007; Takai *et al.*, 2004a). They convert H_2 and CO_2 from degassing magma and water-rock reactions into CH_4 and H_2O. The abundances of methanogens and the extent of methanogenic fluid chemistry alteration are elevated in ultramafic vent sites where serpentinization leads to high H_2 concentrations (Perner *et al.*, 2007; Takai *et al.*, 2004a) and at volcanic eruption sites where H_2 is also more abundant (Holden *et al.*, 1998; Huber *et al.*, 2002b; Von Damm, 2004) (Table 3.2). In contrast, methanogen abundances and impacts are low in mid-ocean ridge and volcanic

arc hydrothermal systems where H_2 concentrations are low (Nakagawa *et al.*, 2006; Takai *et al.*, 2008b; Takai *et al.*, 2009; Ver Eecke *et al.*, 2009) (Table 3.2).

This pattern of H_2-limited methanogenesis fits those proposed in bioenergetic models (Hoehler, 2004), but maintenance energy estimates for these models are scarce above 50°C and lacking above 65°C (Tijhuis *et al.*, 1993). Without a measured threshold, H_2 availability cannot be definitively claimed as a key factor driving methanogen abundance and distribution. The purposes of this study were to define the kinetics and minimum H_2 concentration for the growth of *Methanocaldococcus*, a genus of hyperthermophilic methanogens found at deep-sea vents, and correlate these with field microbiology and fluid geochemistry results from two hydrothermal vent sites in the northeastern Pacific Ocean and other reported vent sites. Our results show that methanogenesis at 70-82°C ceased below 17-23 μM H_2, a minimum threshold that largely explains the *Methanocaldococcus* distribution pattern observed globally in deep-sea vents.

3.3 Materials and Methods

3.3.1 Growth Media

Methanogen medium *'282mod'* is based on DSMZ Medium 282 (www.dsmz.de) and is composed of the following per liter: 0.14 g K_2HPO_4, 0.14 g $CaCl_2 \cdot 2\ H_2O$, 0.25 g NH_4Cl, 3.40 g $MgSO_4 \cdot 7\ H_2O$, 4.10 g $MgCl_2 \cdot 6\ H_2O$, 0.33 g KCl, 0.50 mg $NiCl_2 \cdot 6\ H_2O$, 0.50 mg $Na_2SeO_3 \cdot 5\ H_2O$, 30.0 g NaCl, 1 g $NaHCO_3$, 0.63 g $Na_2S_2O_3$, 5ml 0.01%

(wt vol^{-1}) $Fe(NH_4)_2(SO_4)_2$ • 6 H_2O, 10 ml 141 trace elements, 10 ml 141 vitamins, and 50 µl 5% (wt vol^{-1}) resazurin solution. The medium was pH balanced to 6.00 ± 0.05. The headspace contained 2 atm H_2:CO_2 (80:20). The medium was reduced with 32 µM dithiothreitol. (Jones *et al.*, 1983a; Miller *et al.*, 1988)

Methanogen medium *'399'* is based on DSMZ Medium 399 and is composed of the following per liter: 0.34 g KCl, 4.0 g $MgCl_2$ • 6 H_20, 3.45 g $MgSO_4$ • 7 H_20, 0.25 g NH_4Cl, 0.14 g $CaCl_2$ • $2H_20$, 0.14 g K_2HPO_4, 18.0 g NaCl, 1.0 g $NaHCO_3$, 0.05 g yeast extract, 2.7 g Na_2SO_4, 1.0 g sodium acetate, 5ml 0.01% (wt vol^{-1}) $Fe(NH_4)_2(SO_4)_2$ • 6 H_2O, 10 ml 141 trace elements, 10 ml 141 vitamins, and 50 µl 5% (wt vol^{-1}) resazurin solution. The medium was pH balanced to 6.90 ± 0.05. The headspace contained 2 atm H_2:CO_2 (80:20). The medium was reduced with 0.025% (wt vol^{-1}) each cysteine-HCl and Na_2S • $9H_2O$ prior to inoculation. (Burggraf *et al.*, 1990b)

Heterotroph medium *'141mod'* is composed of the following per liter: 18 g NaCl, 4 g $MgCl_2$ • $6H_2O$, 3.45 g $MgSO_4$ • $7H_2O$, 0.335 g KCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2$ • $2H_2O$, 0.14 g K_2HPO_4, 1.0 g $NaHCO_3$, 5.0 g casein hydrolysate, 2.0 g yeast extract, 1.0 g elemental sulfur, 10 ml 141 trace elements, 10 ml 141 vitamin solution, 1 ml 0.2% (wt vol^{-1}) each Fe $(NH_4)_2(SO_4)_2$ and $(NH_4)_2Ni(SO_4)_2$, 0.1 ml 0.1% (wt vol^{-1}) each Na_2WO_4 • $2H_2O$ and Na_2SeO_4, and 50 µl 5% (wt vol^{-1}) resazurin solution. The medium was pH balanced to 6.80 ± 0.05. The headspace contained 2 atm argon. The medium was reduced with 0.025% (wt vol^{-1}) each cysteine-HCl and Na_2S• $9H_2O$ prior to inoculation. (Holden *et al.*, 1998)

Iron reducer medium *'DLFe'* is composed of the following per liter: 19 g NaCl, 9 g $MgCl_2$ • $6H_2O$, 0.15 g $MgSO_4$ • $7H_2O$, 0.3 g $CaCl_2$, 0.5 g KCl, 0.42 g KH_2PO_4, 0.1 g

$(NH_4)_2SO_4$, 0.05 g KBr, 0.02 g $SrCl_2 \cdot 6H_2O$, 0.5 ml Wolfe's vitamin solution, 1 ml Wolfe's mineral solution, 0.02 g yeast extract, and 100 mM Fe(III) oxide hydroxide. The medium was pH balanced to 6.80 ± 0.05. The headspace contained 2 atm H_2:CO_2 (80:20). The medium was reduced with cysteine-HCl (0.5 mM) and $FeCl_2$ (1.3 mM) prior to inoculation. (Kashefi *et al.*, 2002)

Heterotroph and methanogen co-culture medium *'399-141'* is composed of the following per liter: 0.34 g KCl, 4.0 g $MgCl_2 \cdot 6\ H_20$, 3.45 g $MgSO_4 \cdot 7\ H_20$, 0.25 g NH_4Cl, 0.14 g $CaCl_2 \cdot 2H_20$, 0.14 g K_2HPO_4, 18.0 g NaCl, 1.0 g $NaHCO_3$, 0.05 g yeast extract, 2.7 g Na_2SO_4, 1.0 g sodium acetate, 5.0 g casein hydrolysate, 2.0 g yeast extract, 1.0 g elemental sulfur, 10 ml 141 trace elements, 10 ml 141 vitamins, 1 ml 0.2% (wt vol^{-1}) each $(NH_4)_2Fe(SO_4)_2$ and $(NH_4)_2Ni(SO_4)_2$, 0.1 ml 0.1% (wt vol^{-1}) each $Na_2WO_4 \cdot 2H_2O$ and Na_2SeO_4, and 50 µl 5% (wt vol^{-1}) resazurin solution. The medium was pH balanced to 6.85 ± 0.05. The headspace contained 2 atm N_2:CO_2 (80:20). The medium was reduced with 0.025% (wt vol^{-1}) each cysteine-HCl and $Na_2S \bullet 9H_2O$ prior to inoculation.

3.3.2 Field Sampling: Fluid Chemistry and Microbial Analyses

Using the deep-sea research submarine *Alvin*, samples were collected in the summers of 2008 and 2009 from the Main, High Rise, and Mothra vent fields along the Endeavour Segment and from the caldera of Axial Volcano, both on the Juan de Fuca Ridge in the northeastern Pacific Ocean (Figure 3.1). Hydrothermal fluids (233 total) ranging in temperature from ~2°C (background seawater) to 358°C were collected with a hydrothermal fluid sampler (HFS) with simultaneous fluid temperature measurements at

the intake of the device during sampling (167 samples) and titanium gas-tight samplers that maintain seafloor hydrostatic pressure (66 samples) (Table 3.1 and Table 3.3). Five actively venting sulfide chimneys were recovered and those that were large enough were subsampled into interior and exterior portions (Table 3.4).

The concentrations of H_2, CH_4, and Mg^{2+} (a conservative ion that is absent from pure hydrothermal vent fluid) were determined for each sample as previously described (Butterfield *et al.*, 2004). H_2 concentrations in pure hydrothermal fluids were estimated by extrapolating H_2 concentrations in high-temperature gas tight fluids to zero Mg^{2+}. Subsamples were taken directly from the collapsible bags of the HFS into syringes without exposure to air for shipboard analysis of H_2 and CH_4 using gas chromatography. If a gas headspace was present, then the gas phase and liquid phase volume were measured, and both phases were sampled and analyzed by gas chromatography. Piston samples intended primarily for gas analysis were extracted on the same shipboard gas extraction line used for titanium gas-tight samplers, and the gases sealed for later analysis. The extracted water (acidified with sulfamic acid) was analyzed for major elements on shore. Magnesium ions were analyzed at the Pacific Marine Environmental Laboratory in Seattle as previously described (Butterfield *et al.*, 1997). (Table 3.1)

Twenty-eight of the HFS fluid samples and 8 black smoker chimney samples were used for most-probable-number (MPN) estimates to determine the relative abundances of hyperthermophilic methanogens (*282mod, 399,* and *141mod* media), sulfur-reducing heterotrophs (*141mod* medium), and autotrophic iron oxide reducers (*DLFe* medium) (Table 3.3 and 3.4). To prepare inocula, 50 ml of diffuse hydrothermal fluid was transferred into anoxic sealed serum bottles, and 12-24 g of sulfide material

was added to serum bottles containing 50 ml of sterile, anoxic artificial seawater (www.dsmz.de/media/med141.htm). Inocula were reduced with 0.025% (wt vol^{-1}) each of cysteine-HCl•H_2O and Na_2S•$9H_2O$ and flushed with argon. Three-tube MPN analyses (Holden *et al.*, 1998) were performed by adding 3.33 ml, 0.33 ml, and 0.03 ml of each sample in triplicate to each media. After inoculation, the tubes were incubated at 55°C (2008 methanogens), 85°C (all 2008 metabolisms, 2009 methanogens and 2009 heterotrophs), or 95°C (2009 iron reducers) for up to 7 days. Growth in the tubes was confirmed for all metabolisms using phase-contrast light microscopy. Growth of dissimilatory iron reducers was determined spectrophotometrically by testing for the production of iron(II) using the Ferrozine method (Lovley & Phillips, 1986). Growth of methanogens and H_2-producing heterotrophs was determined by gas chromatography detecting CH_4 and H_2, respectively.

Select positive methanogenic enrichments from 2008 were used as inocula to isolate pure cultures. Isolation was performed by three rounds of 10-fold dilution-to-extinction series incubated at the original enrichment temperatures in 50 ml of *399* medium. Pure strains denoted as JH123 and JH146, were isolated at 70°C and 85°C from fluid samples collected from Axial Volcano sites Cloud and Marker 113, respectively. DNA from each isolate was extracted from 50 ml of culture using a genomic DNA purification kit (Wizard Genomic DNA purification kit, Promega) with the addition of 0.1 mg proteinase K during cell lysis. DNA quantity and purity was checked using a Nano-drop spectrometer. The universal archaeal 16S rRNA primers 21f (5′-TTC CGG TTG ATC CYG CCG GA-3′) and 958r (5′-YCC GGC GTT GAM TCC AAT T-3′) were used for DNA amplification using PCR. The PCR products were

purified and sequenced in both directions using 21f and 958r as the primers. These sequences were then compared with other known nucleotide sequences in the NCBI database using a BLAST analysis (Altschul *et al.*, 1990). Similar sequences were aligned using Silva alignment software (Pruesse *et al.*, 2007) and phylogenetic trees were created using Mega4 software (Tamura *et al.*, 2007).

3.3.3 *Methanocaldococcus* Growth Kinetics Under Varying [H_2]

A 2 liter bioreactor with gas flow, temperature (± 0.1°C), and pH (± 0.1 pH unit) controls was prepared with 1.7 liters of *282mod* medium. The reactor was sparged through a submerged fritted bubbler with 30 ml of argon gas min^{-1} overnight while the temperature was maintained at either 82°C or 70°C and the pH was maintained at 6.8 with 0.25 M HCl. The gas flow was then changed to a mixture of CO_2 (7.5 ml of gas min^{-1}), H_2, and Ar with a cumulative flow rate of 37.5 ml of gas min^{-1}. The H_2 gas flow rate varied for different growth kinetics experiments: 1.4 min^{-1} (10 µM), 2.3 ml min^{-1} (16.5 µM), 3 ml min^{-1} (22 µM), 6 ml min^{-1} (45 µM), 12 ml min^{-1} (90 µM), 24 ml min^{-1} (180 µM), and 30 ml min^{-1} (225 µM). The aqueous H_2 concentration in the reactor at all flow rate settings and both incubation temperatures was measured by drawing 25 ml of fluid from the reactor directly into anoxic serum bottles and measuring the headspace with a gas chromatograph. Argon was added to balance the total gas flow at 37.5 ml of gas min^{-1}, which was found to be optimal in a separate comparative growth kinetic experiment. To reduce the medium, 8.5 ml of 0.2% (wt vol^{-1}) $(NH_4)_2Fe(SO_4)_2$ and 17 ml of 320 mM dithiothreitol were added, and the reactor equilibrated for 1-2 h. Prior to inoculation an additional 8.5 ml of 320 mM dithiothreitol were added to fully reduce the

medium so that the resazurin cleared. 0.25 M HCl was used to pH balance the medium to 6.0. The reactor was then inoculated with 25 ml of a logarithmic-growth-phase culture. During growth, samples were taken from the reactor and cell densities were determined using phase contrast microscopy and a Petroff-Hausser counting chamber. Each growth kinetic experiment was run in duplicate or triplicate.

3.3.4 Co-culturing of Hyperthermophilic Heterotroph and Methanogen

To test for commensalism, *Thermococcus* sp. strain CL1 isolated from a vent site near Axial Volcano (Holden *et al.*, 2001) and *Methanocaldococcus* sp. strain JH146 from Marker 113 at Axial Volcano were grown in *399-141* medium at 82°C. A co-culture was established and maintained through multiple transfers. Growth kinetics experiments were conducted with concomitant H_2 and CH_4 measurements for each strain grown individually and in co-culture. For co-culture growth kinetic experiments, Balch tubes containing 10 ml of media were inoculated with 0.1 ml of logarithmically growing co-culture and 0.05 ml of logarithmically growing methanogen pure culture. During growth, duplicate tubes were permanently removed from incubation at various time points. Once cooled to room temperature, the volume of gas within each tube was measured with a pressure-lock syringe. Gas chromatography was used to measure the total amount of CH_4 and H_2 calculated in each tube.

For visualization of cellular interactions, subsamples of co-culture experiments were analyzed with florescence *in situ* hybridization (FISH) using the probes Tcoc164 (CAV RCC TAT GGG GGA TTA GC) and MC504 (GGC TGC TGG CAC CGG ACT TGC CCA) for *Thermococcales* and *Methanocaldococcaceae*, respectively, as described

previously (Crocetti *et al.*, 2006; Rusch & Amend, 2004). Samples were prepared for FISH by fixing in a 3:1 volume of formaldehyde, incubating overnight at 4°C, pelleting and washing with PBS, pelleting and resuspending in 95% ethanol, fixing onto a gelatin-coated slide, and dehydrating by passing through 50%, 80%, and 95% ethanol. Prehybridization was performed by incubating the slides with hybridization buffer (0.9 M NaCl, 20 mM TRIS, 0.1% (wt vol^{-1}) sodium dodecyl sulfate (SDS), 0-55% formamide) lacking probe at 48°C for 1 h. Samples were then hybridized by incubation with hybridization buffer mixed with 50 ng of probe at 48°C for 3 h. After washing with pre-warmed wash buffer (0.9 M NaCl, 20 mM TRIS (pH 8), 5 mM EDTA, 0.1% (wt vol^{-1}) SDS), and ddH_20, the samples wer mounted and the cells were visualized using epifluorescence microscopy.

3.3.5 Comparing Growth of Hyperthermophilic Iron(III) Oxide Reducer and Methanogen

Growth kinetics experiments were performed with *Methanocaldococcus* sp. strain JH146 and *Hyperthermus* sp. strain Ro04 (Ver Eecke *et al.*, 2009) at 82°C and 95°C, with *399* and *DLFe* media, respectively. Balch tubes containing 10 ml of media were inoculated with a logarithmic-growth-phase culture. During incubation, duplicate tubes were removed from the incubator at various time points and allowed to cool to room temperature. The headspace volume within each tube was measured with a pressure-lock syringe and H_2 and CH_4 were measured by gas chromatography. The concentration of methanogens was determined using phase contrast microscopy and a Petroff-Hausser counting chamber. The concentration of iron reducers was determined by epifluorescence microscopy of samples that were solubilized with an oxalate solution (28

g l^{-1} ammonium oxalate and 15 g l^{-1} oxalic acid), filtered on 0.2 μm black polycarbonate filters, and stained with acridine orange (Daley & Hobbie, 1975).

3.4 Results

3.4.1 *Methanocaldococcus* Growth Kinetics Under Varying [H_2]

M. jannaschii as well as the field isolates, which were found to be phylogenetically related to *Methanocaldococcus* species (Figure 3.2), all had longer doubling times and lower maximum cell concentrations with decreasing H_2 concentrations (Figure 3.3). The lower H_2 threshold for *M. jannaschii* was between 17 μM and 23 μM (Figure 3.3A) while the thresholds for strains JH146 and JH123 were between 10 μM and 17 μM (Figure 3.3B and C). The H_2-dependent growth kinetics for all three strains (Figure 3.3D) were statistically the same despite variations in source location, longevity in pure culture, and incubation temperature suggesting that these data are broadly applicable to *Methanocaldococcus* above 70°C. The K_m and minimum doubling time based on a Lineweaver-Burke plot through all of the data (Figure 3.3D) are 67 μM and 28 min.

3.4.2 Field Sampling: Fluid Chemistry and Microbial Analyses

Maximum H_2 concentrations in hydrothermal end-member fluids were significantly higher at Axial Volcano (up to 424 μmol kg^{-1}) relative to those from the Endeavour Segment (up to 131 μmol kg^{-1}) (Figure 3.4A). Extrapolation of maximum

end-member H_2 concentrations (Table 3.1) to 82°C through conserved mixing with seawater indicates that H_2 concentrations would be in the range of 78-110 μmol kg^{-1} and 19-33 μmol kg^{-1} at Axial Volcano and the Endeavour Segment, respectively (Table 3.2). Based on our pure culture studies, H_2 concentrations are above the predicted K_m necessary for growth at much of Axial Volcano but towards the lower H_2 threshold at the Endeavour Segment (Figure 3.4A). Maximum CH_4 concentrations in fluids from Axial Volcano largely followed conserved seawater-hydrothermal fluid mixing (Figure 3.4B). The exception was the anomalously high CH_4 concentrations found in fluids collected from venting at Marker 113 suggesting that there was extensive methanogenesis occurring in the subseafloor at this site. No CH_4 anomalies were observed in Endeavour Segment hydrothermal fluids (data not shown).

At Axial Volcano, hyperthermophilic methanogens were cultured from only 1 of the 10 low-temperature fluids, which came from the Marker 113 site (table S1), and from the interior of a black smoker chimney collected at El Guapo vent (Tables 3.3 and 3.4). Attempts at growing methanogens at 55°C in 2008 only resulted in one positive enrichment also from the Marker 113 site. At the Endeavour Segment, hyperthermophilic methanogens were cultured from only 2 of the 18 low-temperature fluids (Table 3.3), which came from S & M and Boardwalk vents, and in 3 of the 6 black smoker chimneys collected (Table 3.4). Hyperthermophilic heterotrophs were found in all but one of the MPN enrichments and on average were found at concentrations that were more than 100-fold higher than the hyperthermophilic methanogens (Tables 3.3 and 3.4). Hyperthermophilic autotrophic iron oxide reducers were found in 9 of the 28 low-temperature fluid samples (Table 3.3) and in all 8 of the sulfide samples (Table 3.4) and

on average were at twice the concentration of methanogens. The MPN results mirror those reported previously for the Endeavour Segment (Ver Eecke *et al.*, 2009). In several of our MPN enrichments, methanogens were found growing in co-culture with hyperthermophilic heterotrophs where no H_2 had been added initially (Table 3.3) suggesting commensal growth between the two organisms.

3.4.3 Co-culturing of Hyperthermophilic Heterotroph and Methanogen

The results of growing *Thermococcus* sp. strain CL1 with and without *Methanocaldococcus* sp. strain JH146 show H_2 consumption and CH_4 production in the mixed culture bottles relative to H_2 production alone (Figure 3.5A). This supports the idea that hyperthermophilic heterotrophs are supplying hyperthermophilic methanogens with H_2, especially in H_2 limited systems. FISH staining and microscopy for *Thermococcus* and *Methanocaldococcus* species in our co-cultures suggest that these organisms grow in aggregates where the heterotrophs significantly outnumber the methanogens (Figure 3.5B).

3.4.4 Comparing Growth of Hyperthermophilic Iron(III) Oxide Reducer and Methanogen

A comparison of growth and H_2 consumption rates for *Methanocaldococcus* sp. strain JH146 at 82°C and *Hyperthermus* sp. strain Ro04 at 95°C showed ~200 µmol of H_2 being consumed by the iron reducer during its ~20 hours of logarithmic growth, and ~1,500 µmol of H_2 being consumed by the methanogen during its ~6 hours of logarithmic growth (Figure 3.6). This shows that iron the amount of H_2 consumed per cell mass

produced was far less for the iron reducer than the methanogen supporting the idea that they can grow on far less H_2.

3.5 Discussion

The minimum H_2 concentration for growth of our hyperthermophilic methanogens, 17-23 μM, is more than three orders of magnitude higher than the 7-10 nM minimum H_2 concentration reported for mesophilic methanogens in aquifers and anoxic sediments (Lovley & Goodwin, 1988; Lovley *et al.*, 1994). This difference is in keeping with the predicted species-independent increase in maintenance energies that double with every 9°C increase in growth temperature (Tijhuis *et al.*, 1993). The rate of DNA depurination and deamination is 3,000-fold higher at 100°C than it is at 37°C (Lindahl, 1993), and the higher H_2 demand of *Methanocaldococcus* may reflect a high repair cost of DNA and other cell components at high temperatures. The effect of pressure on our minimum H_2 threshold concentration is uncertain. Pressure has a very minor effect on values of standard molal Gibbs free energy of formation (ΔG°) for reactions occurring in the aqueous phase (Amend & Shock, 2001; Hoehler, 2004). Growth rates and hydrogenase activities of *M. jannaschii* increase with pressure (Boonyaratanakornkit *et al.*, 2007; Miller *et al.*, 1989), but it is not known whether pressure significantly affects the organism's affinity for H_2.

Measured H_2 concentrations were above the predicted K_m necessary for hyperthermophilic methanogenesis at much of Axial Volcano but towards the lower H_2

threshold at the Endeavour Segment, suggesting that hyperthermophilic methanogens are hydrogen limited at some Axial Volcano and most Endeavour Segment venting sites. The MPN estimates of this study correlate with fluid chemistry analyses in that methanogens were scarce but were detected where hydrogen or methane concentrations were elevated. The only positive methanogenic enrichments from the Endeavour Segment fluid samples came from S & M and Boardwalk vents, which had end-member H_2 concentrations of 131 and 115 μmol kg^{-1}, respectively, and were among the most H_2 rich vents at the Endeavour Segment. The majority of positive methanogenic enrichments from Axial Volcano were from the Marker 113 vent site, which was the single site of anomalously high CH_4 concentrations. Fluids from the Marker 113 vent site have previously been found to contain active methanogens (Huber *et al.*, 2009). The Marker 113 vent site is unusual in that it consists entirely of low-temperature (*i.e.*, diffuse) venting out of basaltic rock with the closest high temperature venting more than 730 m away. This is in contrast to most diffuse venting that is typically just meters away from focused high temperature fluid flow and possibly hosts only a relatively thin and shallow subsurface biotic zone. Marker 113 lies between the Coquille and International District vent fields where a volcanic eruption occurred in 1998 (Figure 3.1). These fields have end-member venting with H_2 concentrations up to 376 μmol kg^{-1} and 460 μmol kg^{-1}, respectively. Therefore, possible factors leading to the anomalously high methane concentrations at Marker 113 relative to the other Axial Volcano diffuse vents may be a volumetrically larger subsurface biotic zone heated by a deep magma source that runs between the Coquille and International District vents and a longer residence time of hydrothermal fluids within this zone below Marker 113. The biogenic CH_4 observed at

Marker 113 is an amalgamation of all methanogenesis above the sample temperature (27°C).

The minimum H_2 concentration for hyperthermophilic methanogen growth determined in this study also appears to hold for other systems around the world, regardless of geologic setting, depth, or maximum temperatures (Table 3.2). Mariner Field at Lau Basin, TOTO Caldera in the Mariana Arc, and Brothers Volcano in the Kermadec Arc all had pure hydrothermal fluid H_2 concentrations that would result in H_2 concentrations below our threshold at 82°C, and no methanogens were found at those sites (Nakagawa *et al.*, 2006; Takai *et al.*, 2008b; Takai *et al.*, 2009; Ver Eecke *et al.*, 2009). In contrast, Iheya North Field, Kairei Field, and Logatchev Field had pure hydrothermal fluid H_2 concentrations between 200 and 5,900 μM, and methanogens were found at these sites in direct proportion to the concentration of H_2 present within the system (Nakagawa *et al.*, 2005; Takai *et al.*, 2004a). The high concentrations of hyperthermophilic heterotrophs, which have the same growth temperature and pH ranges as hyperthermophilic methanogens, detected within the same samples (Table 3.2) suggest that temperature and pH were not factors in hyperthermophilic methanogen distributions. Therefore, H_2 limitation appears to be broadly applicable as a general explanation of hyperthermophilic methanogen distribution in deep-sea hydrothermal systems.

Our findings of field enrichments containing methanogens with hyperthermophilic heterotrophs where no H_2 had been added initially and of a laboratory developed co-culture of a H_2 producing heterotroph and a H_2 consuming methanogen greatly suggest commensal growth between these two groups of organisms. Hyperthermophilic methanogens have previously been shown to grow solely on the H_2

produced by hyperthermophilic heterotrophs, which was proposed to ameliorate growth inhibition by H_2 in the latter organisms (Boonyaratanakornkit *et al.*, 2007; Johnson *et al.*, 2006). Our visualization of a seemingly hydrogen syntrophic co-culture shows the heterotrophs greatly outnumbering the methanogens, which may be indicative of the high hydrogen concentrations required for hyperthermophilic methanogenesis. Similar outnumbering by heterotrophs was revealed in the MPN results of this study that estimate heterotrophs being 100 times more abundant than methanogens. Based on kinetic experiments of growth and metabolic rates of *Thermococcales* species (Oslowski *et al.*, *submitted*), it would take a minimum of 40 heterotrophic cells to produce hydrogen at the 17-23 µM scale that would support 1 methanogenic cell. Hydrogen supplied by heterotrophs may allow methanogens to maintain low level populations, nearing the microbial detection limit, in environments with hydrogen limiting conditions. Once conditions change to more favorable hydrogen concentrations, such as after a magmatic event, this low level but active methanogen population can then bloom as seen previously (Holden *et al.*, 1998; Huber *et al.*, 2002b; Von Damm, 2004). If the heterotrophs are supporting the methanogens in low hydrogen vents, then it is likely that both organisms are dependent upon organic compounds that flow in from mesophilic autotrophic bacteria and macrofauna that colonize the outer surfaces of black smoker sulfides (often only a few cm away in our samples). This commensalism would then be spatially constrained by the availability of organic carbon.

Not all hyperthermophilic hydrogenotrophic autotrophs are likely to be H_2 limited in hydrothermal systems such as those found at the Endeavour Segment. For example, the free energy available from the H_2 reduction of ferrihydrite to magnetite ($H_{2(aq)}$ + 6

$Fe(OH)_3 \rightarrow 2\ Fe_3O_4 + 10\ H_2O_{(l)}$) at 100°C is -303.34 kJ per mole of H_2 while the free energy for methanogenesis at the same temperature is -45.25 kJ per mole of H_2 (Amend & Shock, 2001; Holden *et al.*, 2011). Based on thermodynamic predictions, dissimilatory iron reduction would become H_2 limited at a much lower H_2 concentration than methanogenesis, as previously observed for mesophilic microorganisms (Lovley & Goodwin, 1988). Our results of growth and H_2 consumption rates for *Methanocaldococcus* sp. strain JH146 and the iron reducer *Hyperthermus* sp. strain Ro04 show that the amount of H_2 consumed per cell mass produced was far less for the iron reducer than the methanogen, suggesting that it might also have lower maintenance energy costs. Results in this and another study (Ver Eecke *et al.*, 2009) show that hyperthermophilic H_2-oxidizing iron reducers are more abundant than hyperthermophilic methanogens within black smoker chimneys. Therefore, iron reducers appear to out-compete methanogens in low H_2 and mildly reducing vent environments.

While there are several factors that can limit the growth of hyperthermophiles in deep-sea hydrothermal vents (*e.g.*, temperature, pH, ORP, nitrogen availability), the results of this study demonstrate that one of the key determinants for the presence of *Methanocaldococcus* species is H_2 availability at concentrations of at least 17-23 µM. This value will also likely vary within an organism with differences in maintenance energy costs under varying environmental conditions, which is a much needed area of future research. Our results also point to the importance of commensalism, and perhaps syntrophy, between hyperthermophile species. Ultimately, our ability to model the growth and metabolite production rates of microbes with different metabolisms at various temperatures will enable us to make better predictions regarding the habitability and

bioenergetics of various regions of the subseafloor and the microbial impact on carbon flux and chemical cycling within the deep sea and the ocean crust.

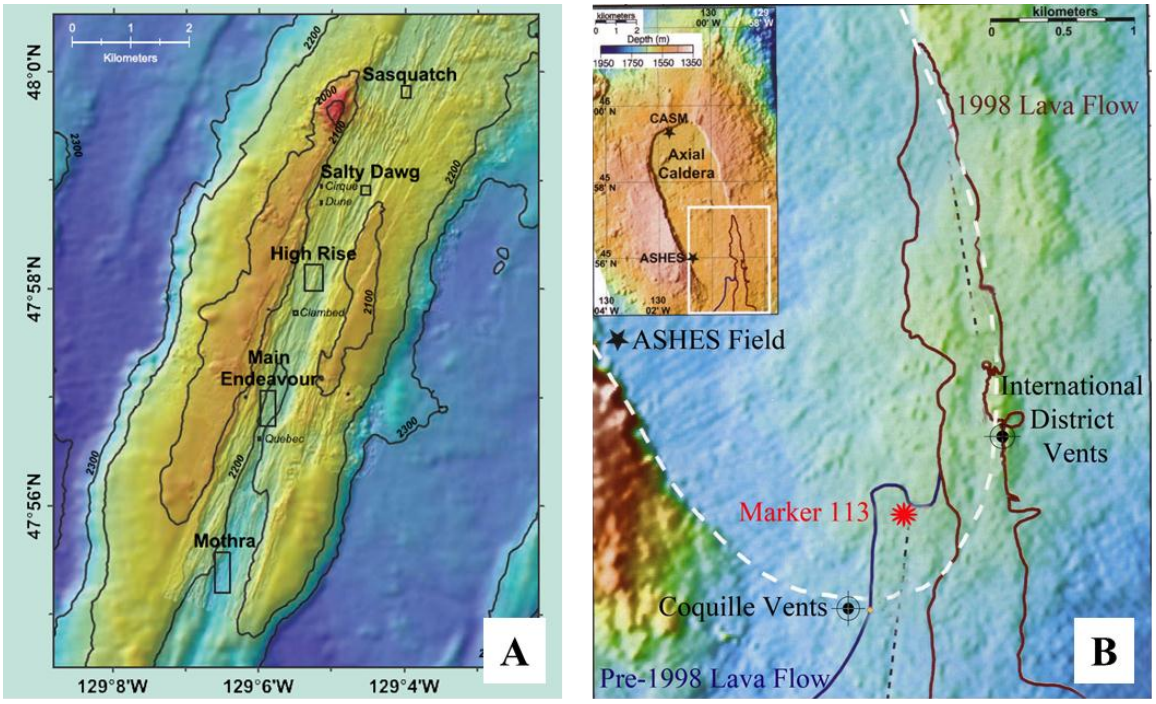

FIGURE 3.1. Bathymetry maps of the hydrothermal vent fields of the Endeavour Segment (A) (Glickson *et al.*, 2007) and Axial Volcano (B) (modified from Butterfield *et al.*, 2004) on the Juan de Fuca Ridge in the northeastern Pacific Ocean.

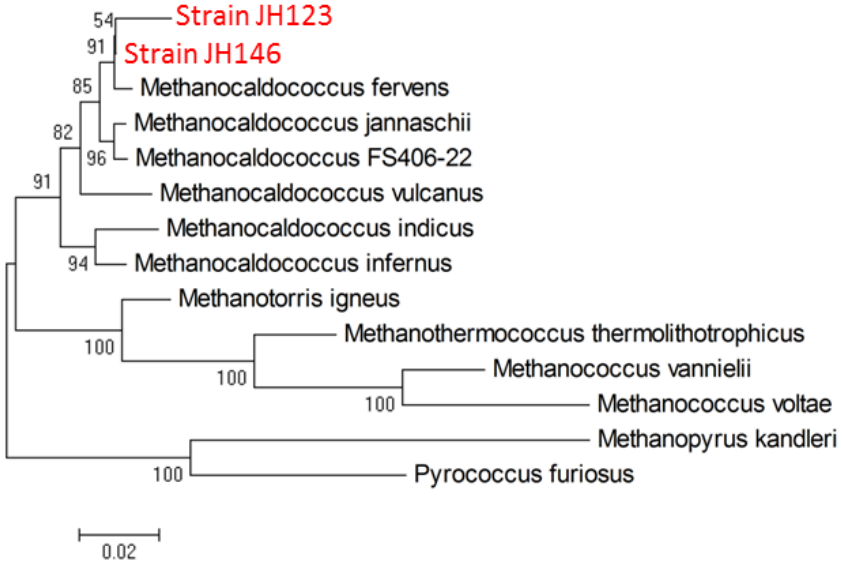

FIGURE 3.2. Phylogenetic analysis of partial 16S rRNA sequences of isolated strains JH123 and JH146 and related species.

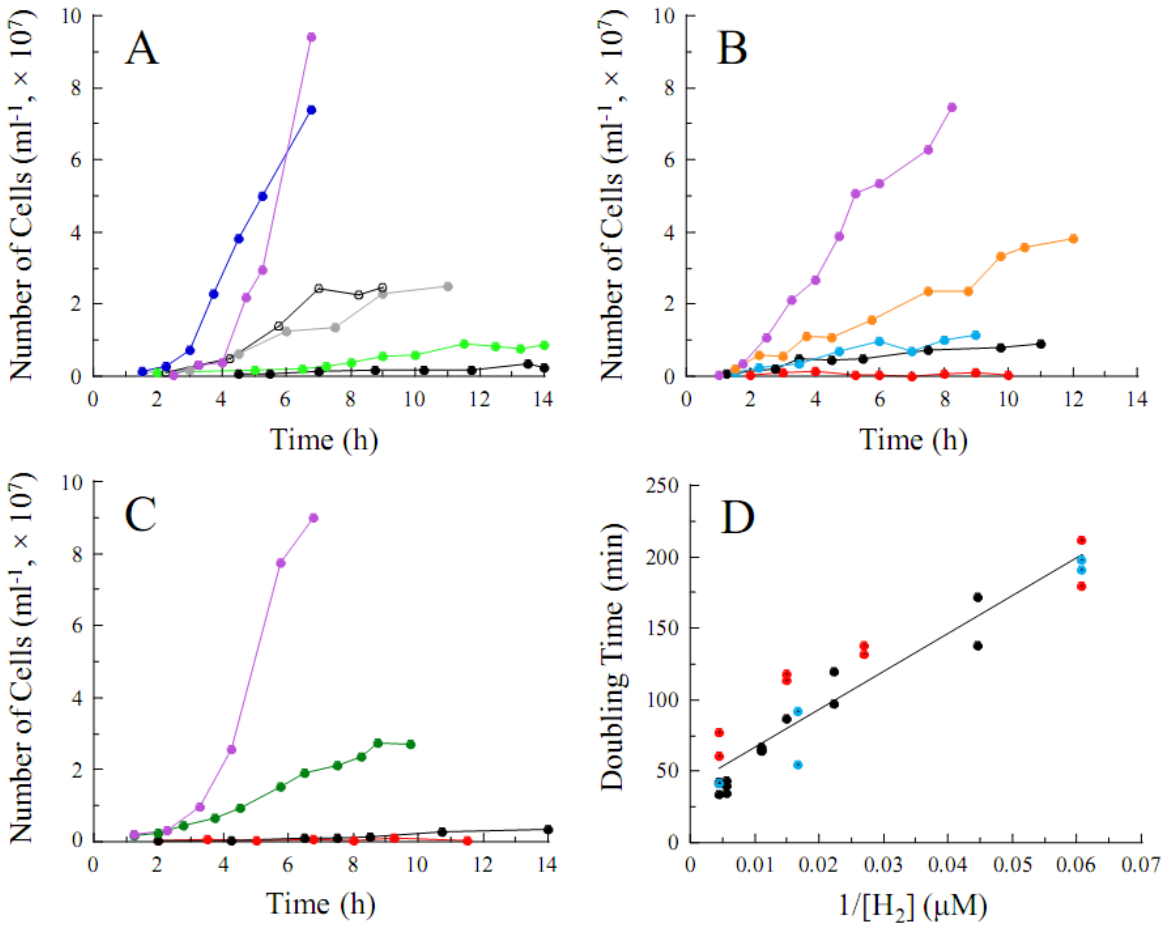

FIGURE 3.3. Growth of *M. jannaschii* (A) and *Methanocaldococcus* strain JH146 (B) at 82°C and *Methanocaldococcus* strain JH123 at 70°C (C) at H_2 concentrations of 10 μM (●), 16.5 μM (●), 22.5 μM (●), 37 μM (●), 45 μM (●), 60 μM (●), 67 μM (●), 90 μM (○), 180 μM (●), and 225 μM (●). (D) A Lineweaver-Burke plot of all the growth data for *M. jannaschii* (●), strain JH146 (●), and strain 123 (●).

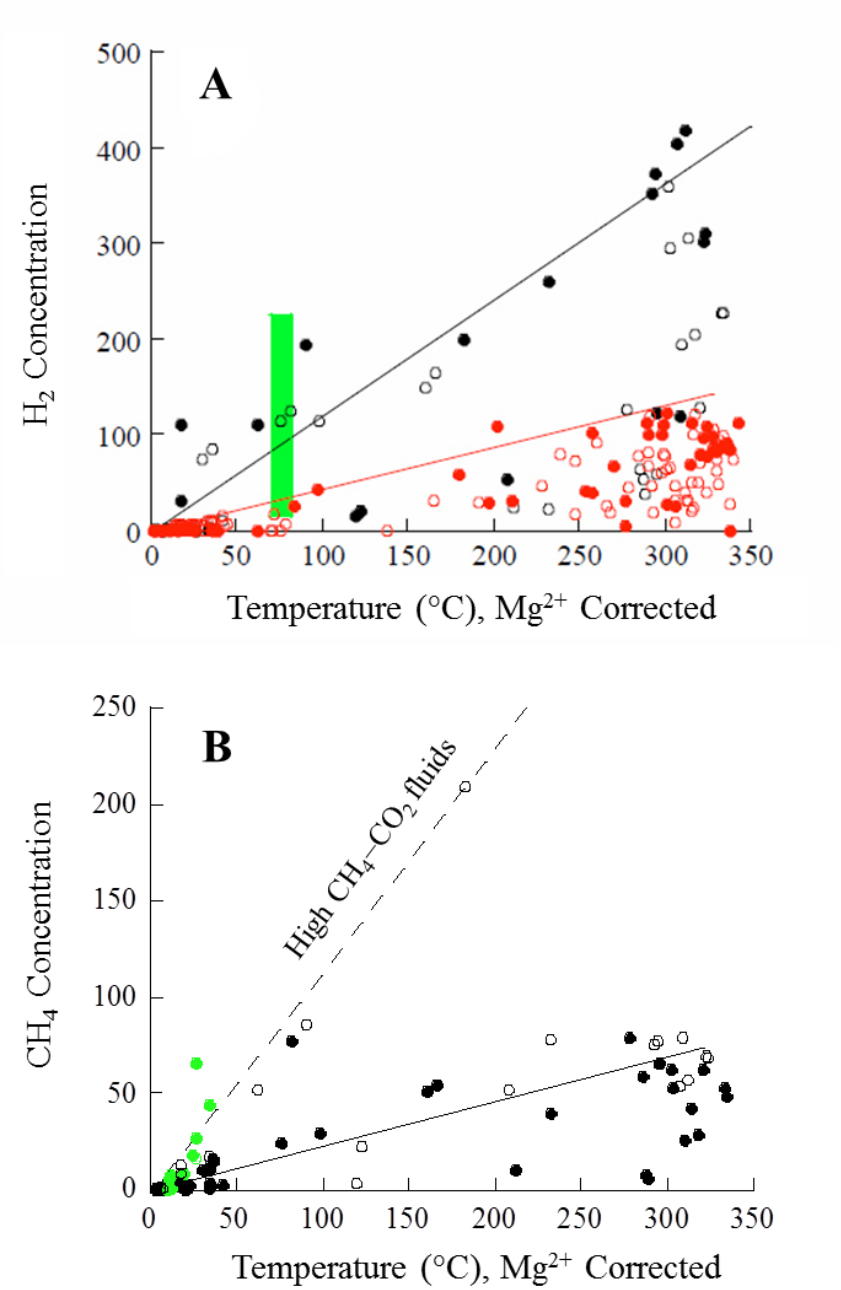

FIGURE 3.4. (A) H_2 concentrations in hydrothermal fluids collected at various temperatures from Axial Volcano (●,○) and the Endeavour Segment (●,○). The green box represents the H_2 concentrations between 70°C and 82°C that support *Methanocaldococcus* spp. growth as shown in Figure 3.3. (B) CH_4 concentrations in hydrothermal fluids collected from Axial Seamount only. The green symbols in B (●,○) are the CH_4 concentrations for fluids from Marker 113. The dashed line shows end-member mixing for the high CH_4-CO_2 fluids at Virgin Mound and Marker 151. For both A and B, filled symbols are for HFS samples (μM), open symbols are for gas tight samples (μmol kg^{-1}), and lines are for end-member mixing between seawater and the hydrothermal fluid with the highest average measured gas concentration in gas tight samples (see Table 3.1).

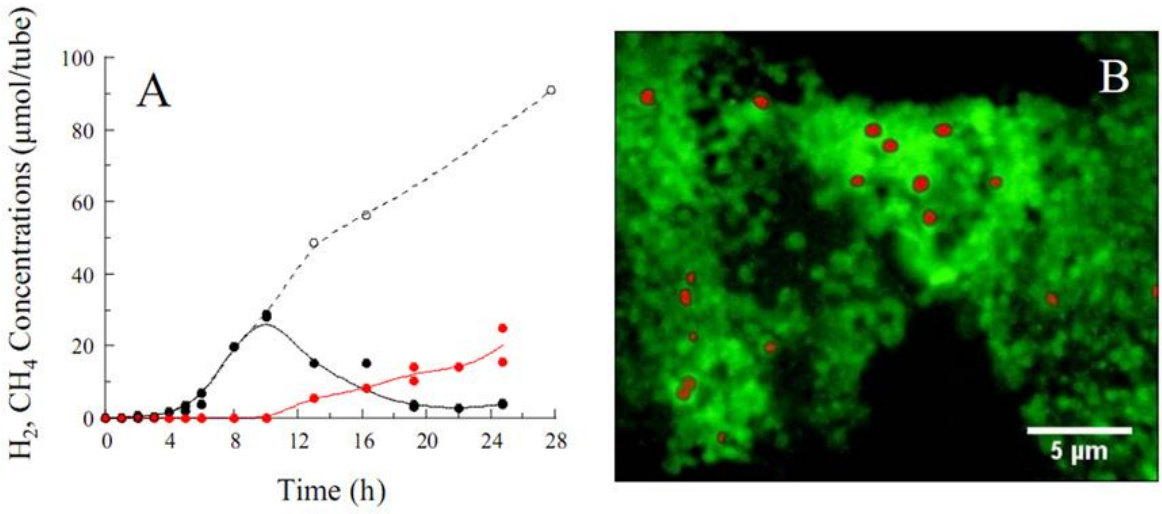

FIGURE 3.5. (A) H_2 production by *Thermococcus* strain CL1 when grown at 82°C with (●) and without (○) *Methanocaldococcus* strain JH146, and CH_4 production by *Methanocaldococcus* strain JH146 (●) when grown in co-culture with strain CL1. (B) Fluorescence *in-situ* hybridization staining of strains CL1 (green) and JH146 (red) grown in co-culture.

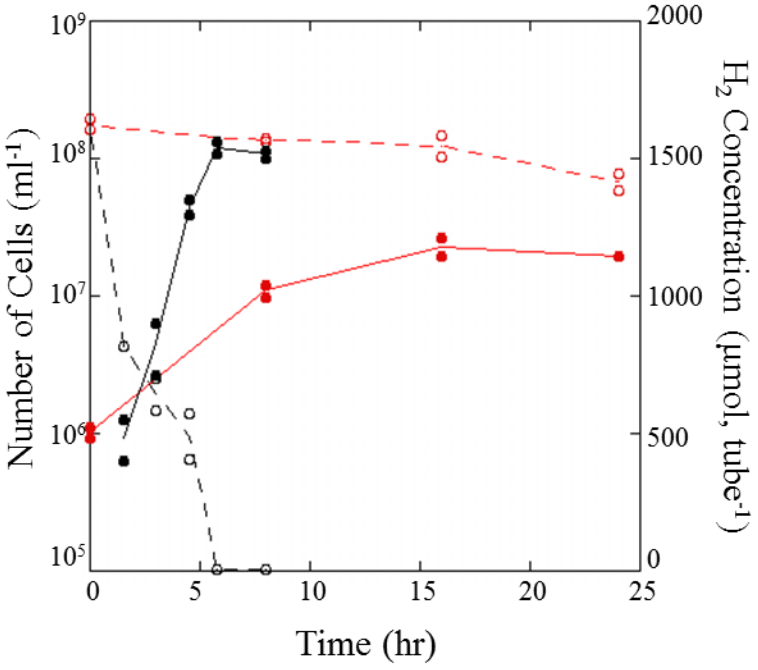

FIGURE 3.6. Growth (solid symbol) and H_2 consumption (open symbol) of *Methanocaldococcus* sp. JH146 (black) and *Hyperthermus* sp. RoO4 (red).

TABLE 3.1. Maximum fluid temperatures and average hydrothermal end-member concentrations of H_2 and CH_4 at various vent sites in 2008 and 2009 from gas tight fluid samples

Vent name	Max. fluid temp. (°C)	End-member gas concentration (μmol kg^{-1}, ± s.d.)	
		H_2	CH_4
Axial Volcano – ASHES:			
Inferno	321	111.4 ± 49.0	73.7 ± 12.8
Hell	286	73.7	71.7
Virgin Mound	248	271.8	284.9
Axial Volcano – Coquille:			
Vixen	334	317.3 ± 5.8	71.7 ± 0.9
Casper	304	375.8 ± 12.8	79.3 ± 0.6
Axial Volcano – International District:			
El Guapo	351	424.3 ± 5.4	57.1 ± 1.6
Castle	246	275.5	83.1
Diva	225	460.8	187.5
Marker 151	170	340.5 ± 42.1	154.3 ± 12.4
Endeavour Segment – Main Endeavour Field:			
Hulk	328	141.6 ± 33.2	1,645.6 ± 189.8
Crypto	328	110.7	1,993.5
Lobo	331	108.7	1,488.7
Grotto	340	70.3 ± 18.5	1,703.8 ± 310.6
TP	335	107.3 ± 8.7	1,914.3 ± 49.9
Dante	338	87.6 ± 2.6	1,545.9 ± 32.6
Dudley	319	75.1	1,926.8
S&M	329	132.5 ± 2.7	2,090.4 ± 2.0
Bastille	317	24.2 ± 12.6	1,582.1 ± 824.4
Sully	257	39.1	2,046.9
Puffer	252	38.5	1,875.0
Cathedral	212	2.7	1,368.4
Milli-Q	72	0.1	1,428.4
Salut	268	41.1	2,159.5
Endeavour Segment – High Rise:			
Godzilla	358	91.0 ± 6.6	4,154.6 ± 516.1
Boardwalk	352	120.9 ± 35.3	3,838.3 ± 578.2
Park Place	332	99.9	3,049.9
Baltic	306	100.1	3,164.0
Ventnor	332	79.6	3,240.2
Fairy Castle	329	82.0	2,264.2
Endeavour Segment – Mothra:			
Cauldron	299	103.2	1,470.6
Cuchalainn	303	117.7	2,023.5
Stonehenge	310	114.5	1,979.8

*Fluid concentrations (μM) from the hydrothermal fluid sampler. Due to gas leakage, these values should be considered minimum estimates of gas concentrations.

TABLE 3.2. Characteristics of various global deep-sea hydrothermal vent sites and abundances of hyperthermophilic methanogens and heterotroph *(Higashi et al., 2004; Holden et al., 1998; Nakagawa et al., 2005; Takai et al., 2009)*(This Study)

	Axial Volcano, Juan de Fuca Ridge	Endeavour Segment, Juan de Fuca Ridge	Mariner Field, Lau Basin	TOTO Caldera, Mariana Arc	Brothers Volcano, Kermadec Arc	Iheya North Field, Mid-Okinawa Trough	Kairei Field, Central Indian Ridge	Logatchev Field, Mid-Atlantic Ridge
Geologic setting[a]	MORB	MORB	VA	VA	VA	VA	UM	UM
Approx. depth (m)	1,520	2,200	1,900	2,900	1,670	1,000	2,400	2,970
Max. temperature of fluid (°C)	334-351	299-358	284	170	290	311	365	350
H_2 conc. (μM) in end-member fluids[b]	97-367	30-128	46	~10	17	~200	2,500	5,900
Estimated H_2 conc. at 82°C (μM)[c]	28-99	10-33	13	5	5	~50	550	1,382
Hyperthermophilic methanogens (max.)	1,290 L^{-1}	690 L^{-1}, 34 g^{-1}	n.d.	n.d.	n.d.	650 g^{-1}	2,500 g^{-1}	+
Hyperthermophilic heterotrophs (max.)	>33,000L^{-1}	>72,000 L^{-1}, 7,200 g^{-1}	10^7 g^{-1}	10^7 g^{-1}	10^7 g^{-1}	3.5×10^7 g^{-1}	6×10^6 g^{-1}	NA

[a]Symbols and abbreviations: MORB, mid-ocean ridge basalt; VA, volcanic arc; UM, ultramafic; +, *Methanocaldococcus*-related 16S rRNA comprised more than 10% of the total archaeal DNA clones analyzed from the sample; n.d., none detected; NA, not available. [b]End-member H_2 and CH_4 concentrations in hydrothermal fluids are based on an extrapolation of measured values to zero magnesium concentration.[c]H_2 concentrations at 82°C are estimated assuming conserved mixing between 2°C seawater with zero H_2 and end-member hydrothermal fluid H_2.

TABLE 3.3. Most-probable-number estimates (MPNs) of hyperthermophilic autotrophic iron reducers, methanogens, and heterotrophic sulfur reducers in diffuse hydrothermal fluids

Sample ID	Sample description	Most-probable-number estimates					Total cell concentration[b]
		Autotrophic Fe(III) reducers	Methanogens, 282 medium	Methanogens, 399 medium	Methanogens, 141 medium	Heterotrophic sulfur reducers	
Endeavour Segment:							
Sa08-1	Salut vent, MEF (43°C)	ND	ND	-	-	33,000 L^{-1}	-
EI08	Easter Island vent, MEF (13°C)	ND	ND	-	-	330 L^{-1}	1.3×10^8 L^{-1}
SM08	S&M vent, MEF (42°C)	2,790 L^{-1}	690 L^{-1}	-	-	13,800 L^{-1}	2.6×10^9 L^{-1}
Lo08	Lobo vent, MEF (31°C)	450 L^{-1}	ND	-	-	>72,000 L^{-1}	1.8×10^9 L^{-1}
Hu08	Hulk vent, MEF (15°C)	270 L^{-1}	ND	-	-	7,200 L^{-1}	2.6×10^8 L^{-1}
Gr08	Grotto vent, MEF (16°C)	270 L^{-1}	ND	-	-	8,700 L^{-1}	9.9×10^7 L^{-1}
Ca08	Cathedral vent, MEF (24°C)	ND	ND	-	-	90 L^{-1}	2.6×10^7 L^{-1}
Cd08	Cauldron vent, Mothra (32°C)	270 L^{-1}	ND	-	-	1,290 L^{-1}	5.3×10^8 L^{-1}
Gz08	Godzilla vent, High Rise (14°C)	ND	ND	-	-	3,600 L^{-1}	2.6×10^8 L^{-1}
Hu09	Hulk vent, MEF (36°C)	ND	ND	ND	ND	12,000 L^{-1}	1.0×10^8 L^{-1}
EI09	Easter Island vent, MEF (9°C)	ND	ND	ND	ND	2,790 L^{-1}	2.8×10^8 L^{-1}
Lo09	Lobo vent, MEF (8°C)	ND	ND	ND	ND	690 L^{-1}	5.7×10^7 L^{-1}
Gr09	Grotto vent, MEF (18°C)	ND	ND	ND	ND	4,500 L^{-1}	3.6×10^7 L^{-1}
Ph09	Phang vent, Mothra (24°C)	90 L^{-1}	ND	ND	ND	>33,000 L^{-1}	4.4×10^7 L^{-1}
Bw09	Boardwalk vent, High Rise (14°C)	ND	120 L^{-1}	270 L^{-1}	ND	7,200 L^{-1}	7.1×10^7 L^{-1}
FC09	Fairy Castle vent, High Rise (25°C)	ND	ND	ND	ND	12,000 L^{-1}	6.1×10^7 L^{-1}

Ve09	Ventnor vent, High Rise (21°C)	90 L^{-1}	ND	ND	ND	33,000 L^{-1}	4.4×10^8 L^{-1}
Bt09	Baltic vent, High Rise (23°C)	ND	ND	ND	ND	12,000 L^{-1}	2.8×10^7 L^{-1}
Axial Volcano:							
Go08	Gollum vent (21°C)	ND[c]	ND	-	-	1,290 L^{-1}	4.5×10^8 L^{-1}
Cl08	Cloud vent (7°C)	ND	ND	-	-	2,250 L^{-1}	5.3×10^8 L^{-1}
M3308	Marker 33 vent (16°C)	ND	ND	-	-	13,800 L^{-1}	2.0×10^8 L^{-1}
M11308	Marker 113 vent (20°C)	600 L^{-1}	ND[d]	-	-	13,800 L^{-1}	1.6×10^9 L^{-1}
BC08	Bag City vent (11°C)	ND	ND	-	-	ND	1.7×10^8 L^{-1}
M11309-1	Marker 113 vent (34°C)	ND	120 L^{-1}	ND	1,290 L^{-1}	>33,000 L^{-1}	1.1×10^9 L^{-1}
M11309-2	41 m from Marker 113 vent (11°C)	ND	ND	ND	ND	120 L^{-1}	8.1×10^8 L^{-1}
M11309-3	58 m from Marker 113 vent (12°C)	90 L^{-1}	ND	ND	ND	270 L^{-1}	4.5×10^8 L^{-1}
M3309	Marker 33 vent (34°C)	ND	ND	ND	ND	630 L^{-1}	2.9×10^8 L^{-1}
Cl09	Cloud vent (6°C)	ND	ND	ND	ND	2,790 L^{-1}	5.3×10^8 L^{-1}

[a]Estimates are per liter of fluid collected.
[b]The concentration of cells in background seawater was 2.5×10^7 L^{-1}.
[c]ND, not detectable.
[d]There were 120 methanogens L^{-1} that grew at 55°C in this sample.

TABLE 3.4. Most-probable-number estimates (MPNs) of hyperthermophilic autotrophic iron reducers, methanogens, and heterotrophic sulfur reducers in black smoker chimneys

Sample ID	Sample description	Most-probable-number estimates[a]				
		Autotrophic Fe(III) reducers	Methanogens, 282 medium	Methanogens, 399 medium	Methanogens, 141 medium	Heterotrophic sulfur reducers
Ba08-1	Bastille chimney, MEF, interior (282°C)[e]	108 g^{-1}	34 g^{-1}	-	-	3,470 g^{-1}
Ba08-2	Bastille chimney, MEF, interior (282°C)	4 g^{-1}	ND	-	-	>886 g^{-1}
Da08	Dante chimney, MEF, interior (300°C)	13 g^{-1}	4 g^{-1}	-	-	511 g^{-1}
HH08	Hot Harold chimney, Mothra, interior (321°C)	106 g^{-1}	ND	-	-	1,061 g^{-1}
Da09-1	Dante chimney, MEF, exterior (336°C)	1,740 g^{-1}	ND	ND	14 g^{-1}	7,200 g^{-1}
Da09-2	Dante chimney, MEF, interior (336°C)	10 g^{-1}	ND	ND	ND	103 g^{-1}
EG09-1	El Guapo chimney, Axial Volcano, exterior (351°C)	11 g^{-1}	ND	ND	ND	>45,200 g^{-1}
EG09-2	El Guapo chimney, Axial Volcano, interior (351°C)	330 g^{-1}	4 g^{-1}	ND	ND	690 g^{-1}

[a]Estimates are per gram of dry weight sulfide material.
[b]Fluid temperatures are for hydrothermal end-member fluids flowing out of the sulfide deposits at the time of collection.
[c]ND, not detectable.

CHAPTER 4

MODELING THE GROWTH AND BIOENERGETICS OF A HYPERTHERMOPHILIC DEEP-SEA METHANOGEN FROM AXIAL VOLCANO

4.1 Abstract

A hyperthermophilic methanogen, strain JH146, was isolated from low-temperature deep-sea hydrothermal fluid collected from Axial Volcano in the northeastern Pacific Ocean and characterized for the purpose of modeling high temperature methanogenesis at this site. Strain JH146 uses hydrogen and carbon dioxide as its sole energy and carbon sources and is closely related to *Methanocaldococcus* species based on 16S rRNA gene sequence analysis. In this study, growth and methane production rates were measured across ranges of temperature (58-90°C), pH (4.5-9.0), NaCl concentration (4.5-54 g l^{-1}), and nitrogen availability. Bioenergetic estimates were derived from these to predict environmental habitability and distribution patterns for hyperthermophilic methanogens. Under non-stressful conditions, methane production rates and growth energies were uniform at 0.16 ± 0.08 pmol CH_4 $cell^{-1}$ $doubling^{-1}$ and $1.52 \times 10^{-14} \pm 0.57 \times 10^{-14}$ kJ $cell^{-1}$ sec^{-1}, respectively. The optimal growth temperature of JH146 was 82°C, and above 85°C methane production rates and growth energies increased with temperature up to 0.98 pmol CH_4 $cell^{-1}$ $doubling^{-1}$ and 6.87×10^{-14} kJ $cell^{-1}$ sec^{-1}, respectively. JH146 did not fix N_2, lacked the N_2-fixing (cluster II) *nifH* gene, and

became nitrogen limited below 0.14 mM NH_4Cl. With decreasing ammonia availability, methane production rates and growth energies increased to 0.72 pmol CH_4 $cell^{-1}$ $doubling^{-1}$ and 8.16 $\times 10^{-14}$ kJ $cell^{-1}$ sec^{-1}, respectively. Low ammonia concentrations (< 10 μM) in hydrothermal fluids at Axial Volcano suggest that hyperthermophilic methanogens are nitrogen limited there unless alternative sources are available. In this study, we report the characteristics of strain JH146 as a means to present an approach to characterization studies that includes measuring growth and metabolic rates simultaneously and calculating bioenergetics under varying environmentally relevant conditions, which are pertinent to quantitative models predicting habitability, distribution patterns, productivity, and carbon flux.

4.2 Introduction

In order to define subseafloor habitability and biogeochemical processes such as carbon flux in deep-sea hydrothermal vents, quantitative models need to be developed that draw upon *in situ* geochemistry and physiology-based bioenergetic predictions of metabolic reactions over a range of environmentally relevant conditions. The purpose of this study was to isolate a deep-sea hyperthermophilic methanogen and model its growth over a wide range of environmental parameters by measuring growth and methane production rates and calculating cell-specific growth energies. Bioenergetic estimates consist of growth energy, maintenance energy, and survival energy. Growth energy is the energy required to create new biological material, maintenance energy is the energy

required to maintain the functionality of a cell independent of biomass production (*e.g.*, osmotic regulation, turnover of macromolecules), and survival energy is the energy required only for the repair of macromolecular damage (Morita, 1997). Bioenergetic models of these parameters have been made for hydrogenotrophic methanogenesis between -20°C and 130°C (Hoehler, 2004). However, bioenergetic estimates for these models are lacking for methanogens and all other anaerobes at temperatures above 65°C (Tijhuis *et al.*, 1993), and the estimates that do exist for thermophiles often overlook the effect of suboptimal environmental conditions. Maintenance energies in the moderate thermophiles *Methanothermobacter thermautotrophicus* and *Methanothermococcus thermolithotrophicus* did increase significantly at 65°C when the organisms switched from growth on NH_4Cl to N_2 reflecting the high energetic cost of N_2 fixation on the cell (Fardeau *et al.*, 1987). This indicates that bioenergetics fluctuate with environmental conditions.

Methanocaldococcus species are hyperthermophilic methanogens and are cosmopolitan members of deep-sea hydrothermal vent communities that have been isolated from vent sites on the East Pacific Rise (Jeanthon *et al.*, 1999a; Jeanthon *et al.*, 1999b; Jones *et al.*, 1983a), the Mid-Atlantic Ridge (Jeanthon *et al.*, 1998), the Central Indian Ridge (L'Haridon *et al.*, 2003), and the Juan de Fuca Ridge (Mehta & Baross, 2006). As part of an effort to model habitability and biogeochemical processes at high temperatures, a new strain of *Methanocaldococcus*, strain JH146, was purified from Axial Volcano and used for this study. Axial Volcano is a submarine volcano on the Juan de Fuca Ridge in the northeastern Pacific Ocean (45° 58′N 130° 0′W) whose summit is 1,520 m deep. One hydrothermal vent site within the Axial summit caldera,

known as Marker 113 vent, shows large amounts of biogenic methane within the fluid and hosts an apparently large (hyper)thermophilic methanogen population within the crust (Butterfield *et al.*, 2004; Huber *et al.*, 2009). Strain JH146 was isolated from fluids at this site, as was the only known hyperthermophilic methanogen that can fix N_2, *Methanocaldococcus* strain FS406-22 (Mehta & Baross, 2006). The goal of this study was to determine the growth and methane production rates and growth energies of this methanogen over its range of growth temperatures, pH, and concentrations of NaCl and NH_4Cl.

4.3 Materials and Methods

4.3.1 Growth Kinetics and Bioenergetic Calculations

The standard medium used was based on DSM medium 399 (Burggraf *et al.*, 1990b) and was composed of the following per liter: 0.34 g KCl, 4.0 g $MgCl_2 \cdot 6\ H_2O$, 3.45 g $MgSO_4 \cdot 7\ H_2O$, 0.25 g NH_4Cl, 0.14 g $CaCl_2 \cdot 2H_2O$, 0.14 g K_2HPO_4, 18.0 g NaCl, 1.0 g $NaHCO_3$, 0.05 g yeast extract, 2.7 g Na_2SO_4, 1.0 g sodium acetate, 10 ml DSM Medium 141 trace elements, 10 ml DSM Medium 141 vitamins, 0.01% (wt vol^{-1}) $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, and 50 µl 5% (wt vol^{-1}) resazurin solution. The medium was pH balanced to 6.90 ± 0.05. The headspace contained 2 atm H_2:CO_2 (80:20). The medium was reduced with 0.025% (wt vol^{-1}) each cysteine-HCl and $Na_2S \cdot 9H_2O$ prior to inoculation. Cultures were grown at 82°C in a forced-air incubator unless otherwise stated.

Growth and methane production on an amended medium was confirmed after three successive transfers on that medium. For kinetic experiments, at least ten Balch tubes containing 10 ml of media were inoculated concurrently with a logarithmic growth phase culture grown under the same experimental conditions. At various time points, at least two tubes were removed from incubation. Once cooled to room temperature, the volume of gas within each tube was measured with a pressure-lock syringe. Gas chromatography was used to measure the amount of CH_4 in an aliquot of the headspace, which was then used to calculate the total amount of CH_4 in each tube. Similarly, the number of cells per tube was calculated using phase contrast microscopy and a Petroff-Hausser counting chamber. It was assumed that all cells under a particular set of growth conditions will produce the same amount of CH_4 per cell for each cell doubling. This rate was determined for each growth experiment from the linear slope of plotting the total amount of CH_4 in each tube against the total number of cells in each tube for each time point in an experiment (Figure 4.1). Cell-specific growth energy (GE) was calculated using the following equation:

$$GE\ (kJ\ cell^{-1}\ sec^{-1}) = CH_4\ production\ rate \times \Delta G_r^\circ \times (doubling\ time)^{-1}$$

The CH_4 production rate (mol CH_4 $cell^{-1}$ $doubling^{-1}$) is measured as described above, the value for ΔG_r° (kJ mol^{-1} of CH_4) was drawn from previously determined temperature-dependent estimates (Amend & Shock, 2001), and the doubling time (sec) was calculated from the growth kinetic experiment (Figure 4.1).

The range of temperatures permitting growth was determined by incubating strain JH146 at temperatures ranging from 45°C to 97°C. The pH range for growth was determined by the addition of 10 mM of various buffers: formic acid, pH 3.5-4.0; malic acid, pH 4.5-5.0; MES, pH 5.5-6.0; PIPES, pH 6.5-7.0; HEPES, pH 7.5; Tris, pH 8.0-8.5; and glycine, pH 9.0-10.0. The pH of the medium was adjusted after heating it at 82°C for 1 h and then cooling to room temperature. Ammonia concentrations were varied from 0.05 mM to 11.75 mM under stringent nitrogen conditions, which included omission of yeast extract, cysteine and $Fe(NH_4)_2(SO_4)_2$ from the medium, acid washing the tubes, and the use of distilled and deionized water. To test for the utilization of nitrogen sources other than NH_4, the growth medium and tubes were stringently stripped of trace nitrogen so that no growth occurred without supplementation with a nitrogen source, as described above. The medium was supplemented with 10 mM KNO_3 or with a headspace containing H_2:N_2:CO_2 (50:30:20). Media subsets were supplemented with molybdenum, tungsten, or vanadium to potentially stimulate nitrogen fixation.

To test for the utilization of carbon sources other than CO_2, the growth medium was supplemented with 5 g l^{-1} formate, 0.5% (vol vol^{-1}) methanol, or 2 g l^{-1} yeast extract with pure H_2 in the headspace. To determine if there was any stimulatory effect of these carbon sources, growth kinetics experiments were performed with media supplemented with the above organics and H_2:CO_2 (80:20) in the headspace. To test utilization of energy sources other than H_2, the media were supplemented with the above organics and the headspace was composed of N_2:CO_2 (80:20). Acetate was omitted from the medium to determine whether acetate was required for or stimulated growth.

4.3.2 Isolation and Phylogenetic Identification

Strain JH146 was originally enriched for at 85°C using low-temperature (26°C) diffuse hydrothermal fluid collected at Marker 113 vent at Axial Volcano using a hydrothermal fluid sampler and the deep-sea research submarine *Alvin* in August 2008. Purification of the strain was performed by three rounds of 10-fold dilution-to-extinction series (using 50 ml of medium in serum bottles).

DNA was extracted from 50 ml of culture using a genomic DNA purification kit (Wizard Genomic DNA purification kit, Promega) with the addition of 0.1 mg proteinase K during cell lysis. DNA quantity and purity was determined using a Nano-drop spectrophotometer. The primers 21f (5′-TTC CGG TTG ATC CYG CCG GA-3′) and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3′) were used to amplify the 16S rRNA gene using the polymerase chain reaction (PCR). The functional gene of methanogenesis, *mcrA,* was PCR amplified with the primers ME1 (5'-GCM ATG CAR ATH GGW ATG TC-3′) and ME2 (5'-TCA TKG CRT AGT TDG GRT AGT-3′) as previously described (Hales *et al.,* 1996). The nitrogen fixation functional gene, *nifH,* was PCR amplified with two primers (5'-GGH AAR GGH GGH ATH GGN AAR TC-3' and 5'-GGC ATN GCR AAN CCV CCR CAN AC-3') as previously described (Mehta *et al.,* 2003). The 16S rRNA - 23S rRNA intergenic spacer region (ITS) was PCR amplified using primers 21f and 64r (5'-GCC NRG GCT TAT CGC AGC TT-3′) (Reysenbach *et al.,* 1992; Summit & Baross, 2001). The PCR products were purified and sequenced in both directions using the amplification primers for that gene. These sequences were then compared with other known nucleotide sequences in the NCBI database using a BLAST analysis (Altschul *et al.,* 1990). Sequence alignments of similar

sequences were made using Silva alignment software (Pruesse *et al.*, 2007), sequence identity matrices were calculated using BioEdit (Hall, 1999), and phylogenetic trees were created using Mega4 software (Tamura *et al.*, 2007).

4.3.3 Light and Electron Microscopy

For negative staining of whole mounted cells, 10 ml of culture within a sealed Balch tube were fixed by adding 0.2 ml 50% glutaraldehyde with gentle agitation and incubating at room temperature for 1 h. An aliquot of the fixed culture (3 ml) was then removed from the sealed Balch tube and added to two 1.5 ml Eppendorf tubes. These were then centrifuged at 2,000 × g for 8 min, resuspended in PBS (8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4, 0.24 g l^{-1} KH_2PO_4), centrifuged again in the same manner, and resuspended in 3% ammonium acetate. Samples were then applied to plasma-treated carbon films (ca. 0.5 nm thickness) on 400 mesh copper grids and incubated in a moist chamber for 5 min. Grids were then rinsed by 2 cycles of incubation with a drop of 3% NH_4OH, draining on filter paper, 15 sec incubation with a drop of 2% aqueous uranyl-acetate (pH 4), and draining on filter paper. Once air dried, the grids were viewed with a JEOL-100S transmission electron microscope.

For thin section microscopy, 10 ml of culture within a sealed Balch tube were fixed by adding 0.4 ml of 50% glutaraldehyde, with gentle agitation, and incubating at room temperature for 2 h. An aliquot of the fixed culture (3 ml) was then removed from the sealed Balch tube and added to two 1.5 ml Eppendorf tubes. These were centrifuged at 2,000 × g for 8 min, resuspended and pooled in PBS, and washed by two additional cycles of centrifugation and resuspension in PBS. The sample was then post fixed in 1%

OsO_4 in PBS for 1 hour at room temperature. After two cycles of centrifugation and resuspension in dH_2O, the samples were then enrobed by resuspending in a minimal volume of 2% type IX agarose (melted and cooled to 25°C) to create a non-friable unit rich in cells. The agarose was then gelled at 4°C for 5 min, and cut into 1 mm blocks with a razor blade. These blocks were then rinsed in dH_2O, dehydrated by 5 min successive passes through 30%, 50%, and 70% ethanol, and stored overnight at -20°C in 70% ethanol. The blocks were then warmed to room temperature and further dehydrated by 5 min successive passes through 80%, 90%, 95%, and 100% ethanol, followed by 100% acetone. The blocks were then infiltrated from acetone with 25%, 50%, 75%, and 100% steps (2 h each) of Ellis-Spurrs low-viscosity epoxy resin formulation (Ellis, 2006). The sample was embedded and polymerized at 60°C for 48 hours. Polymerized blocks were sectioned on a diamond knife set at 60 nm thickness. Sections were stained with 2% aqueous uranyl-acetate for 30 min, rinsed, stained with alkaline lead citrate (5 mg/ml in 0.1N NaOH) for 5 minutes, and rinsed. Sections were viewed on a JEOL 100S TEM.

4.3.4 Antibiotic Susceptibility

The sensitivity of strain JH146 to chloramphenicol (75 μg ml^{-1}), penicillin (200 μg ml^{-1}), streptomycin (200 μg ml^{-1}), kanamycin (200 μg ml^{-1}), ampicillin (22 μg ml^{-1}), and rifampicin (μg ml^{-1}) was tested in triplicate. Growth was confirmed after three successive transfers by measuring the amount of cells and CH_4 after incubation.

4.4 Results

4.4.1 Growth Kinetics and Bioenergetic Calculations

Growth of strain JH146 was observed between 58°C and 90°C (optimum of 82°C, Figure 4.2A), between pH 4.5 and 9.0 (optimum pH 6.0-6.5, Figure 4.2B), between 4.5 g l^{-1} and 54 g l^{-1} NaCl (optimum 18 g l^{-1} NaCl, Figure. 4.2C), and between 0.14 and 9.4 mM NH_4Cl (Figure 4.3A). There was no growth of JH146 when NH_4Cl and all other sources of nitrogen in the medium were replaced with either 10 mM KNO_3 or N_2 in the headspace. When strain JH146 was grown with N_2 as its sole N_2 source, methane production was decoupled from growth (Figure 4.3C). The organism was found to be an obligate hydrogenotrophic autotroph that did not utilize yeast extract, acetate, formate, or methanol as an alternative source of carbon or energy. No growth occurred when the headspace contained either pure H_2 or N_2:CO_2. Furthermore, yeast extract, acetate, methanol, and formate had no stimulatory effect on the growth of JH146 in the presence of H_2 and CO_2 (doubling times of 30 ± 5 min) (Figure 4.4). In contrast, altering the medium to *282mod* (Jones *et al.*, 1983b; Miller *et al.*, 1988), which lacks acetate and other nutrients did decrease the growth rate (68 min doubling time) (Figure 4.4B).

Under non-stressful conditions, methane was produced at a uniform rate (0.16 ± 0.08 pmol CH_4 $cell^{-1}$ $doubling^{-1}$) (Figure 4.2A-C). For these same conditions, growth energies were also consistent (1.52×10^{-14} ± 0.57 $\times10^{-14}$ kJ $cell^{-1}$ sec^{-1}) (Figure 4.2D-F). At 87°C and 90°C, methane production rates and growth energies increased to 0.98 pmol $cell^{-1}$ $doubling^{-1}$ and 6.9 $\times10^{-14}$ kJ $cell^{-1}$ sec^{-1}, respectively, indicating that the organism was stressed and compensated for this by increasing its rate of metabolism (Figure

4.2A,D). When NH_4 concentration was varied, the growth rate did not change significantly between NH_4Cl concentrations (24.5 ± 5 min doubling time). However, the methane production rates and growth energies increased with decreasing nitrogen availability up to 0.72 pmol $cell^{-1}$ $doubling^{-1}$ and 8.2 $\times 10^{-14}$ kJ $cell^{-1}$ sec^{-1}, respectively (Figure 4.4A,B).

4.4.2 Isolation and Identification

Based on its 16S rRNA gene sequence, strain JH146 grouped phylogenetically with members of the genus *Methanocaldococcus* (Figure 4.5A). Strain JH146 is also phenotypically similar to *Methanocaldococcus* genus in that it has a hyperthermophilic optimum growth temperature (80-90°C), slightly acidic optimum growth pHs (6.0-6.8), and marine optimum growth salt concentrations (15-30 g l^{-1}). The 16S rRNA sequence of JH146 is most closely related to that of *Methanocaldococcus* strain FS406-22 (99.3% identity) (Table 4.1), which was also isolated from the Marker 113 vent at Axial Volcano. However, our data show that strain JH146 differs phenotypically from *Methanocaldococcus* strain FS406-22 in that it cannot fix N_2. Accordingly, the *nifH* gene sequence of strain JH146 is closely related to other *Methanocaldococcus* cluster IV *nifH* genes, which is the non-functional N_2 fixing *nifH* gene in these organisms (Figure 4.5B). It was 96.8% identical to *M. jannaschii nifH* and 94.5% identical to the cluster IV *nifH* gene from *Methanocaldococcus* strain FS406-22. In contrast, strain JH146's *nifH* gene was only 60.7% similar to the cluster II *nifH* gene from *Methanocaldococcus* strain FS406-22, which is the functional N_2 fixing gene (Table 4.1). Like most *Methanocaldococcus* species, there was no evidence for a cluster II *nifH* in strain JH146.

The m*crA* gene sequence from JH146 was most closely related to that of *M. vulcanius* (97.9% identity) and was 93.3% identical to that of *Methanocaldococcus* strain FS406-22 (Figure 4.5C, Table 4.1). The intergenic spacer region sequence of strain JH146 was 98.4% identical to that of *M. jannaschii* and *Methanocaldococcus* strain FS406-22 (Figure 4.5D, Table 4.1).

4.4.3 Light and Electron Microscopy

Microscopic observation revealed irregularly shaped coccoids, 1-2 µm in diameter depending on growth phase. Cells were typically observed as single cells or as doublets. Thin section electron microscopy exhibited a typical archaeal cell envelope consisting of the cytoplasmic membrane and a single surface layer (S-layer) (Figure 4.6A). A polar membrane-like structure was observed underlying the plasma membrane adjacent to flagellar insertion sites, similar to that found in *M. voltae* and *M. jannaschii* (Jones *et al.*, 1983b; Koval & Jarrell, 1987). Inclusion bodies of unknown composition were present in many of the thin sections, which are possibly indicative of intracellular organization or may be artifacts of sample preparation. Since the strain was heavily flagellated, parts of the flagella were observed even on thin sections. Negative staining revealed that flagellation was mainly restricted to one specific region of the cell body, where numerous flagella originated (Figure 4.6B).

4.4.4 Antibiotic Susceptibility

Strain JH146 was resistant to penicillin, streptomycin, kanamycin, and ampicillin, while sensitive to chloramphenicol and rifampicin.

4.5 Discussion

At Axial Volcano in the northeastern Pacific Ocean, high rates of methanogenesis occur at a low temperature vent site known as Marker 113 that are primarily due to the activity of thermophilic and hyperthermophilic methanogens (Butterfield *et al.*, 2004; Huber *et al.*, 2009). The purpose of this study was to model the growth of a hyperthermophilic methanogen isolated from this site over a wide range of environmental parameters by measuring growth and methane production rates and calculating cell-specific growth energies. A *Methanocaldococcus* sp. known as JH146 was purified and shown to grow optimally at 82°C. Phylogenetically, it is most closely related to *Methanocaldococcus* strain FS406-22 (99.3% identity based on 16S rRNA sequences), which was also isolated from Marker 113 (Mehta & Baross, 2006), but unlike this organism, JH146 cannot fix N_2. It is not unusual for *Methanocaldococcus* species to have > 97% 16S rRNA sequence similarity and most were deemed novel based on other comparative techniques. Our sequence similarity results of 16S rRNA, *nifH, mcrA,* and ITS suggest that strain JH146 may be a subspecies of *Methanocaldococcus* strain FS406-22. The phenotypic difference of diazotrophic growth capability was confirmed phylogenetically by strain JH146 only having a cluster IV (non-functional) *nifH.* Cluster II of *nifH* genes is composed of methanogen and bacterial functional nitrogenases including *Methanocaldococcus* strain FS406-22, *M. maripaludis, Methanosarcina* species, *and Methanobacterium* species. Cluster IV is composed of divergent nitrogenases from archaea that cannot fix nitrogen such as *M. jannaschii, M. voltae,* and *M. kandleri.* It is possible that these methanogens have lost the ability to fix nitrogen due

to being in culture with excess ammonia, but the presence of these genes within environmental samples from termite guts, ammonia depleted hydrothermal fluids, and ammonia rich hydrothermal fluids, suggests that there are methanogens in the environment that possess mutated *nifH* genes that no longer encode functional nitrogenases (Mehta *et al.*, 2003).

In the *Methanococcales*, hydrogenotrophic methanogenesis involves Na^+ translocation rather than H^+ translocation (Thauer *et al.*, 2008). Two Na^+ ions are translocated across the cytoplasmic membrane for each CH_4 produced, which yield 0.5 ATP via a Na^+-translocating ATP synthase on the membrane. Since Na^+ ion translocation and ATP synthesis are linked directly with methanogenesis, an increase in CH_4 production rates and growth energies is a likely reflection of an increased demand for ATP. Methane production rates and growth energies were strikingly uniform across the organism's temperature, pH, and salt concentration ranges for growth. The exceptions were at superoptimal growth temperatures and under increasing nitrogen-limiting conditions. These increases indicate that strain JH146 compensated for additional energy requirements triggered by the stressor by increasing its rate of metabolism. Remarkable compensation for nitrogen limitation was observed in that methane production rates and growth energies increased with decreasing NH_4 availability while growth rate remained uniform. This energy may have been required for an ammonia scavenging process. When nitrogen levels were below 0.14 mM NH_4, strain JH146 could no longer compensate and growth became inconsistent and failed, but a low density population was maintained and methane was produced.

Ammonia concentrations in pure end-member hydrothermal fluid at Axial Volcano range from 10-18 μmol kg^{-1} (Butterfield *et al.*, 2004), and therefore, based on our defined lower threshold, *Methanocaldococcus* species are ammonia limited at Axial Volcano unless there is an alternative source of ammonia present. In contrast, at the sedimented coastal hydrothermal vent known as Guaymas Basin, where *Methanocaldococcus* species have been isolated (Jones *et al.*, 1989), ammonia concentrations range from 11-16 mmol kg^{-1} (Von Damm, 1990), which would not limit the growth of methanogens. The environmental pressure of low NH_4 levels at Axial Volcano may have selected for the diazotrophic growth exhibited by *Methanocaldococcus* strain FS406-22. Because the energy cost of nitrogen fixation is one of the highest known for any anabolic process (requiring 16 ATP for each N_2 molecule reduced to 2 NH_3 (Karl *et al.*, 2002)), methanogens that do fix nitrogen do so only when ammonia is limiting and energy sources (*e.g.* H_2) are abundant.

The growth energy calculations in this study provide ground truth to energy conservation estimates for hyperthermophilic methanogens. A previous bioenergetic model predicted cell-specific maintenance energy and H_2 activity requirements for hydrogenotrophic methanogenesis between -20°C and 130°C (Hoehler, 2004), but these estimates were extrapolated to hyperthermophilic conditions because previous data sets lacked information on anaerobes at temperatures above 65°C (Tijhuis *et al.*, 1993). The Hoehler study (2004) estimated that the maintenance energy required for growth at 82°C was $\sim 7\times10^{-13}$ kJ $cell^{-1}$ sec^{-1}. In contrast, our calculated maintenance energy requirement was $1.52\times10^{-14} \pm 0.57 \times10^{-14}$ kJ $cell^{-1}$ sec^{-1} under non-stress inducing growth conditions, which is 47-fold lower than the threshold predicted for minimal growth by Hoehler

(Figure 4.7). Therefore, further refinement of bioenergetics models is warranted for microbial growth at thermophilic and hyperthermophilic temperatures.

In this study we present an approach to microbial characterizations that has potential benefits to understanding the bidirectional relationship between organisms and their environment. We suggest focusing on narrowly defining growth constraints and examining growth and metabolic rates at these thresholds of life, rather than focusing on denoting growth optima. Characterizations were in the past focused on growth optima so as to identify the organism, but now phylogenetics allows us to feel more confident in our identification and permits the incorporation of more intriguing and beneficial questions of our characterization experiments. Narrowly defined growth constraints can be used in predictive models of microbial distribution patterns based on quantifiable thresholds of environmental conditions. Growth and metabolic rates measured under varying environmentally relevant conditions can be used for bioenergetic calculations that can then be coupled with free energy and diffuse flux models to more comprehensively model the growth and impact of methanogens below the seafloor in deep-sea hydrothermal vent systems.

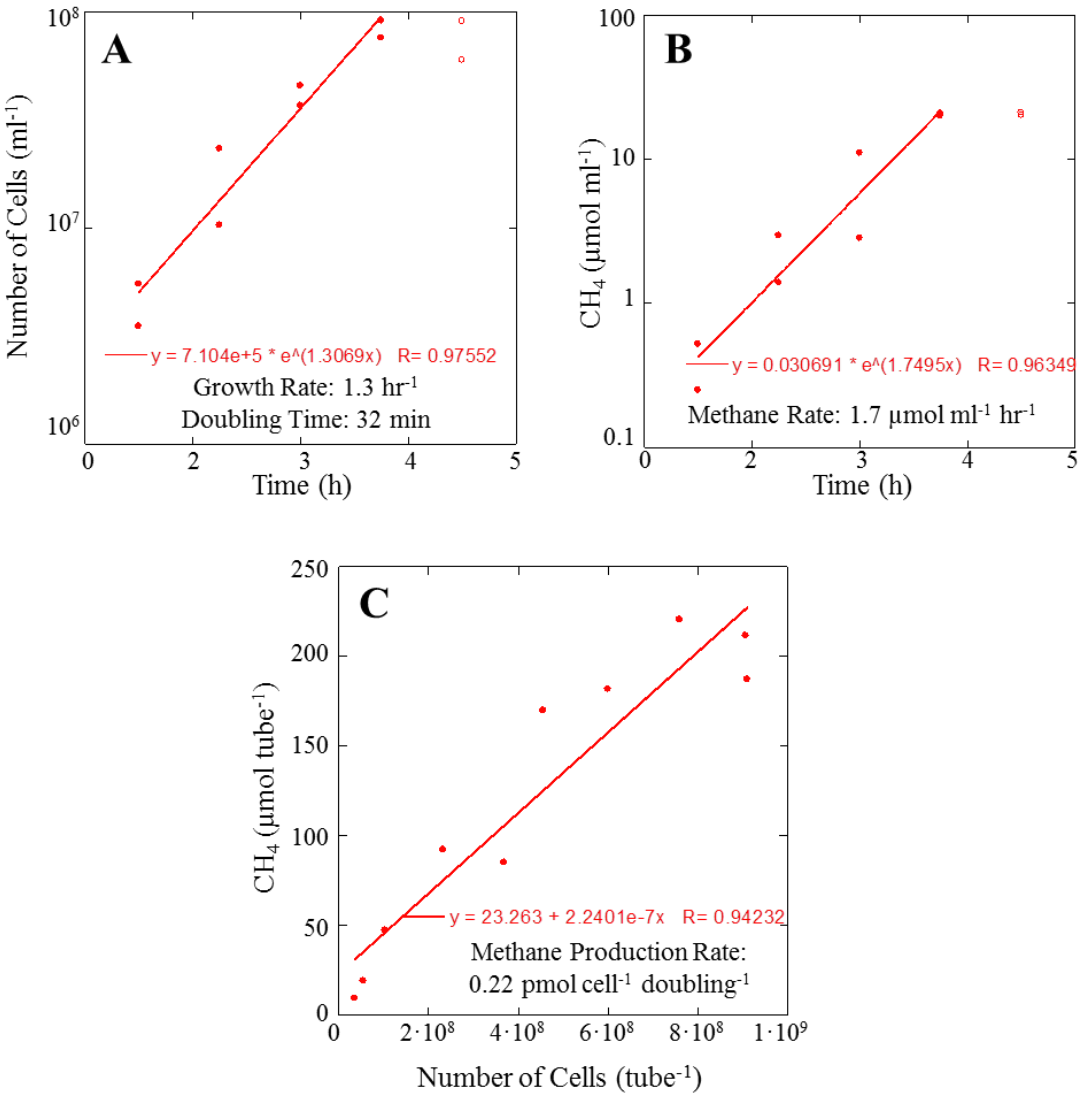

FIGURE 4.1. Example of a growth kinetic experiment determining growth (A) and methane production rates (B) and methane production rate per cell doubling (C).

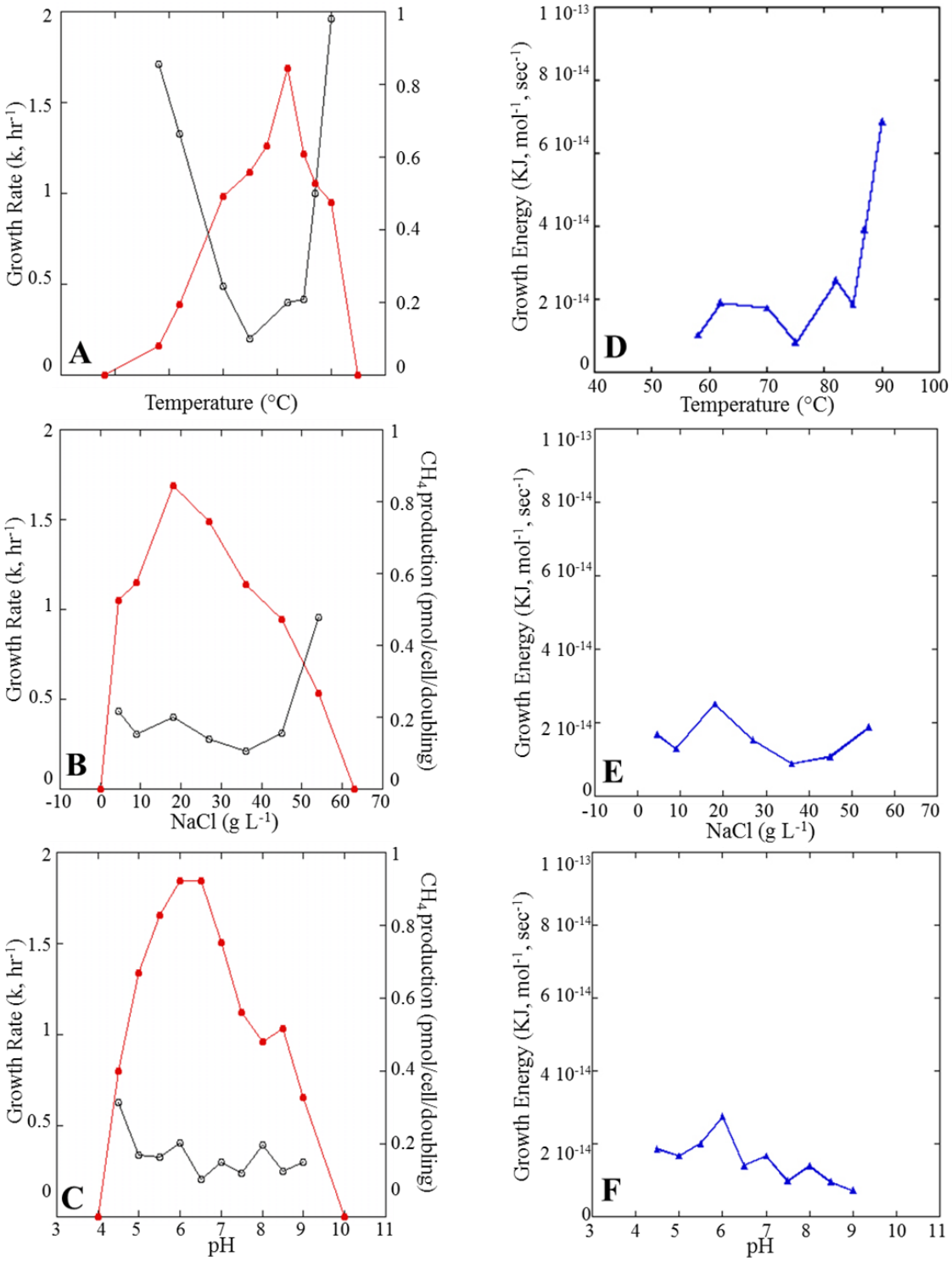

FIGURE 4.2. Growth (●) and methane production (○) rates (A-C) and growth energy calculations (▲) (D-F) of *Methanocaldococcus* sp. JH146 at various temperatures (A, D), pH values (B, E), and NaCl concentrations (C, F).

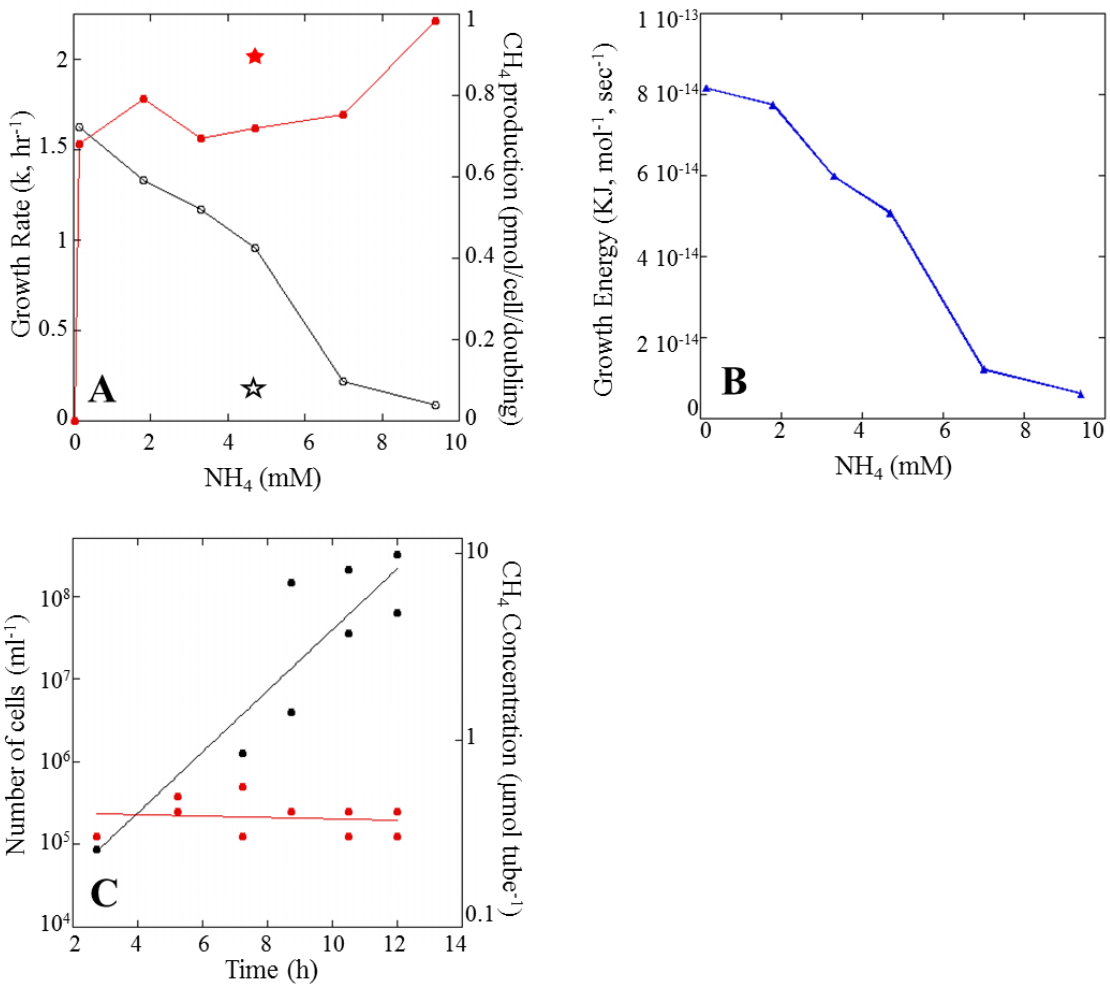

FIGURE 4.3. Growth (●) and methane production (○) rates (A) and growth energy calculations (▲) (B) of *Methanocaldococcus* sp. JH146 at various NH_4 concentrations. (C) Cell (●) and methane (●) concentrations when *Methanocaldococcus* sp. JH146 was incubated with N_2 as the sole nitrogen source.

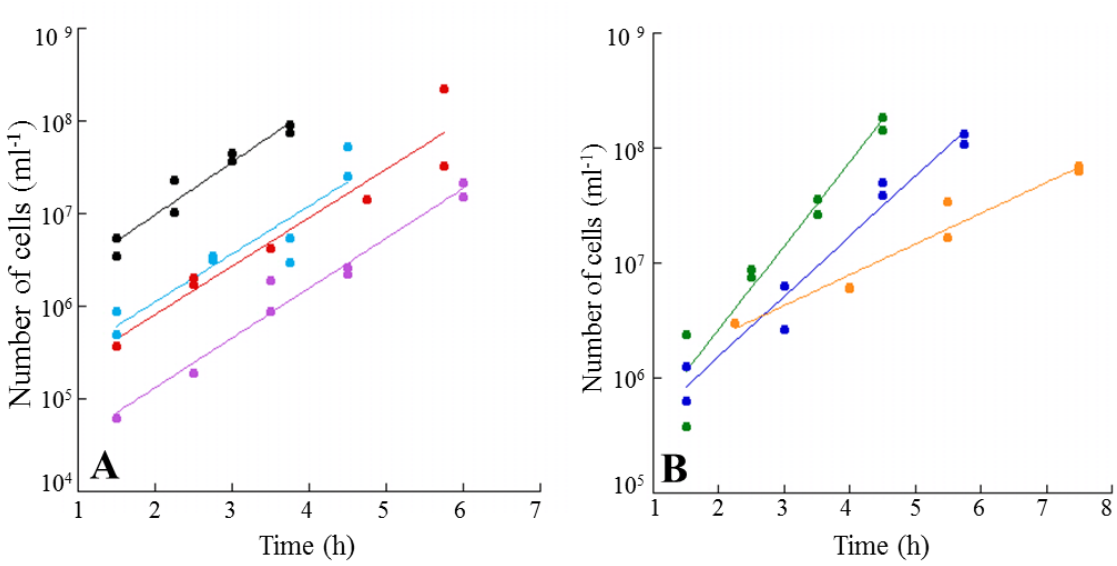

FIGURE 4.4. Growth of *Methanocaldococcus* strain JH146 with media conditions varying in organics. (A) Shows growth with 399 media without the addition of organics (●), and with the addition of 2 g l^{-1} yeast extract (●), 0.5% methanol (●), and 5 l^{-1} formate (●). (B) Shows growth with standard 399 media, which contains acetate (●), with 399 media with acetate omitted (●), and with 282mod media, which lacks acetate and several other nutrients (●).

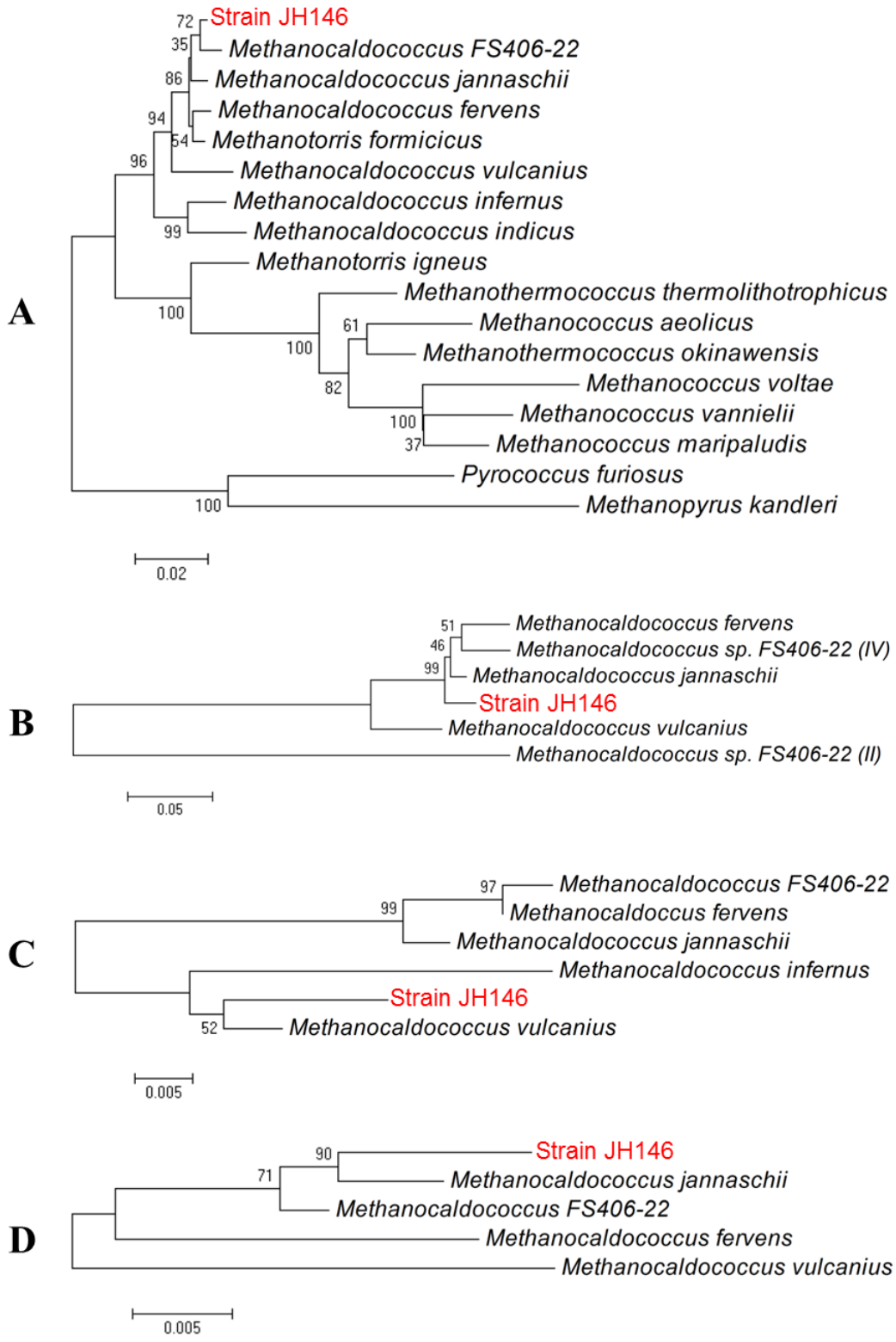

FIGURE 4.5. Phylogenetic sequence analysis of 16S rRNA (A), *nifH* (B), *mcrA* (C), and ITS (D) of *Methanocaldococcus* strain JH146 and related species.

TABLE 4.1. Sequence simiarity matrix of *Methanocaldococcus* strain JH146 compared to other known *Methanocaldococus* gene sequences

	JH146	*M. vulcanius*	*M. infernus*	*M. jannaschii*	*M. fervens*	*M.* FS406-22
16S rRNA						
JH146						
M. vulcanius	0.967					
M. infernus	0.970	0.951				
M. jannaschii	0.990	0.972	0.961			
M. fervens	0.984	0.970	0.956	0.983		
M. FS406-22	0.993	0.965	0.964	0.987	0.977	
M. indicus	0.960	0.950	0.965	0.955	0.948	0.955
nifH						
JH146						
M. vulcanius	0.898					
M. jannaschii	0.968	0.898				
M. fervens	0.945	0.882		0.953		
M. FS406-22 IV	0.945	0.867		0.960	0.945	
M. FS406-22 II	0.607	0.623		0.615	0.600	
mcrA						
JH146						
M. vulcanius	0.979					
M. infernus	0.950	0.962				
M. jannaschii	0.941	0.950	0.929			
M. fervens	0.937	0.945	0.925	0.987		
M. FS406-22	0.933	0.941	0.920	0.983		
ITS						
JH146						
M. vulcanius	0.950					
M. jannaschii	0.984	0.956				
M. fervens	0.962	0.956		0.968		
M. FS406-22	0.984	0.967		0.989	0.968	

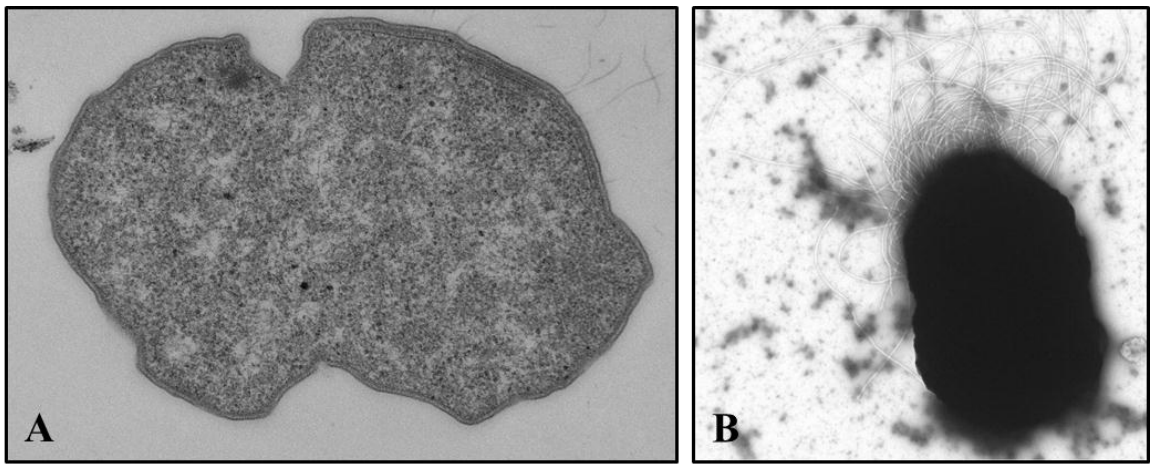

FIGURE 4.6. Thin-section (A) and negative staining (B) electron microscopy of *Methanocaldococcus* strain JH146

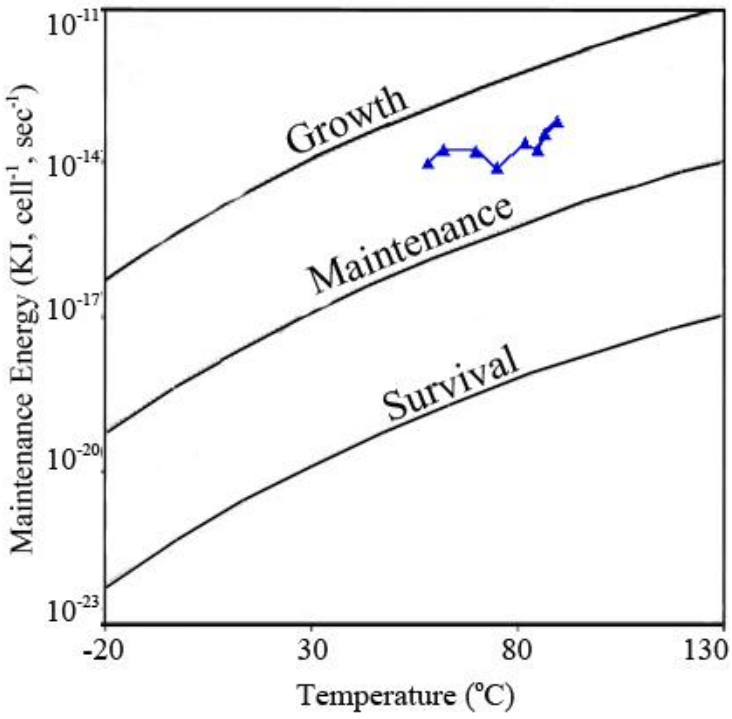

FIGURE 4.7. Calculated cell-specific growth energies of *Methanocaldococcus* strain JH146 at temperatures ranging from 58-90°C (▲) compared to the cell-specific maintenance energies predicted for growth, mainenance, and survival states of hydrogenotrophic methanogens (Hoehler, 2004).

CONCLUSIONS

This dissertation presented three research projects with the goal of 1) determining the metabolic diversity and abundances of hyperthermophiles in deep-sea hydrothermal vents, 2) defining growth kinetics and constraints of hyperthermophilic methanogens in order to relate thresholds of environmental conditions to microbial distribution patterns, and 3) characterizing a hyperthermophilic methanogen so as to model growth and metabolic rates and bioenergetics under varying environmentally relevant conditions. The first and second projects found that autotrophic iron(III) oxide reducers were ubiquitous within samples from the Endeavour Segment and Axial Volcano while methanogens were relatively less abundant and generally absent. The second research project showed that, aside from anomalous sites, hyperthermophilic methanogens are hydrogen limited at the Endeavour Segment and other basalt-hosted, non-eruptive hydrothermal vent sites globally but were not limited at parts of Axial Volcano. This conclusion is based on determining the growth constraints and the effect of hydrogen concentration on the growth kinetics of *Methanocaldococcus jannaschii* and two new field isolates of the same genus. The hydrogen-dependent growth kinetics for all three strains were statistically indistinguishable, exhibiting longer doubling times and lower maximum cell concentrations with decreasing hydrogen concentrations until growth ceased below 17-23 μM. This minimum hydrogen concentration for hyperthermophilic methanogenesis was correlated with field microbiology and fluid geochemistry data, and anomalously high methane concentrations and thermophilic methanogens were only

observed in fluid samples where hydrogen concentrations were above this predicted threshold. As indicated by *in situ* sampling and co-culture experiments, methanogens may depend on hydrogen produced by hyperthermophilic heterotrophs. The third research project fully characterized a *Methanocaldococcus* strain that was included in the hydrogen assessment with an emphasis on measuring growth and metabolic rates simultaneously so as to model bioenergetics. Across the ranges of temperature, pH, NaCl concentration and nitrogen availability, it was found that methane production and growth energy increased under certain stressful conditions and could vary independently from growth rate. This approach of calculating growth energies would facilitate the development of quantitative models predicting habitability, microbial distribution patterns, flux, and cycling within hydrothermal vent systems and the subsurface biosphere. The results of this dissertation demonstrate that the rates and constraints on metabolic processes can be used to predict the distribution and biogeochemical impact of hyperthermophiles in deep-sea hydrothermal vent systems.

The data presented in this dissertation support the hypothesis that there are deterministic microbial distribution patterns based on quantifiable thresholds of environmental conditions. The reported results demonstrate that one of the key determinants for the presence of hyperthermophilic methanogens is hydrogen availability at concentrations of at least 17-23 μM. The hydrogen concentrations of the Endeavour Segment were such that hyperthermophilic methanogens were hydrogen limited while hyperthermophilic autotrophic iron(III) oxide reducers were not, a rational reason for iron(III) oxide reducers being relatively more abundant than methanogens. This quantified threshold correlates with the distribution and abundances of hyperthermophilic

methanogens at other global deep-sea hydrothermal vent sites (Higashi *et al.*, 2004; Holden *et al.*, 1998; Nakagawa *et al.*, 2005; Takai *et al.*, 2009). This is in direct support of the deterministic (niche) theories of biogeography, which have been continually refuted with stochastic (neutral) theories for over a century. Deterministic theories are founded on the idea that "everything is everywhere, but the environment selects," and that "predictive law-like generalizations" have the potential to explain the presence or absence of certain microbes (Baas Becking, 1934; Beijerinck, 1913). Previous microbial studies and models founded in this deterministic theory have shown that mesophilic iron(III) reducers and methanogens would reside in different niches based on different environmental conditions (Bekins *et al.*, 1999; Lovley & Goodwin, 1988; Lovley *et al.*, 1994). It is with this and other previous work in mind that our target hyperthermophilic metabolic groups (autotrophic iron(III) oxide reducers, methanogens, and heterotrophic sulfur reducers) were chosen. The presence of heterotrophic sulfur reducers of the family *Thermococcaceae* is indicative of temperature and pH ranges suitable for both methanogens of the genus *Methanocaldococcus*, and iron(III) oxide reducers of the *Pyrodictiaceae* family. Autotrophic iron(III) oxide reducers being relatively more abundant than methanogens in samples that contained heterotrophic sulfur reducers is indicative of environmental factors other than temperature or pH, such as reduction potential, hydrogen availability, or mineralogy, differentially selecting these autotrophic metabolisms. If the environmental thresholds that determine microbial habitability were quantified then *in situ* tracer models could be developed. An *in situ* tracer is something already present in the system being studied that can be used to infer the various chemical and physical components of the system. *In situ* models based on growth kinetics,

constraints, and mechanisms would lead to a greater understanding of the geological impact microbial reactions have on a planet or moon and the geological signatures they leave behind.

Habitability is a theme throughout the work presented in this dissertation. What are the limits of life that dictate where life can exist? Comparative assessments of growth constraints of different metabolic types have reasoned distribution patterns, but defining the requirements and limits of life in this manner has much broader implications to understanding our planet, other planets or moons, and the origins of life. Hydrothermal vents serve as an outcrop of the subsurface biosphere, about which little is known. The marine subsurface is expected to constitute one of the largest and most widespread reservoirs of biomass on Earth but it is still unknown what kinds of organisms inhabit it, what the habitable dimensions are, what the total biomass is, or what impact these microbes have on global circulations. Likewise, we know little about where and under what circumstances life could have originated or where in our universe it may also have spawned. There are many parallels accepted between deep-sea hydrothermal vents and the prospective habitable regions of moons such as Europa or Enceladus and of early Earth. Hypotheses concerning these prospective habitats or their inhabitants cannot be tested by direct experimentation because we cannot reproduce the unknown environments with certainty, so the analogous environment of deep-sea hydrothermal vents aid in expanding our knowledge on these engaging questions. Compilations of further research using this approach, which aims to narrowly define the growth constraints and model growth kinetics and bioenergetics of hyperthermophiles from deep-sea hydrothermal vents, would further our ability to define life and habitable environments.

BIBLIOGRAPHY

Abrams, R. H., Loague, K. & Kent, D. B. (1998). Development and testing of a compartmentalized reaction network model for redox zones in contaminated aquifers. *Water Resour Res* **34**, 1531-1541.

Adams, M. W. & Kletzen, A. (1996). Oxidoreductase-type enzymes and redox proteins involved in fermentive metabolisms of hyperthermophilic archaea. *Adv Protein Chem* **48**, 101-180.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Amend, J. P. & Shock, E. L. (2001). Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiology Reviews* **25**, 175-243.

Baas Becking, L. G. M. (1934). *Geobiologie of inleiding tot de milieukunde*. The Hague, the Netherlands: W.P. Van Stockum & Zoon.

Barns, S. M., Delwiche, C. F., Palmer, J. D. & Pace, N. R. (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9188-9193.

Baross, J. A. & Hoffman, S. E. (1985). Submarine hydrothermal vents and associated gradient environments as sites for the origin and evolution of life. *Origins of Life and Evolution of Biospheres* **15**, 327-345.

Bauer, M. W., L. E. Driskill, W. Callen, M. A. Snead, E. J. Mathur & Kelly, R. M. (1999). An endoglucanase, EglA, from the hyperthermophilic archaeon Pyrococcus furiosus hydrolyzes β-1,4 bonds in mixed-linkage (1→3),(1→4)-β-D-glucans and cellulose. *Journal of Bacteriology* **181**, 284-290.

Beijerinck, M. W. (1913). *De infusies en de ontdekking der backteriën. Jaarboek van de Koninklijke Akademie voor Wetenschappen*. Amsterdam, the Netherlands: Müller.

Bekins, B. A., Godsy, E. M. & Warren, E. (1999). Distribution of microbial physiologic types in an aquifer contaminated by crude oil. *Microbial Ecology* **37**, 263-275.

Bell, G. (2000). The distribution of abundance in neutral communities *American Naturalist* **155**, 606-617.

Biddle, J. F., Lipp, J. S., Lever, M. A. & other authors (2006). Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3846-3851.

Bischoff, J. L. & Seyfried, W. E. (1978). Hydrothermal chemistry of seawater from 25 degrees to 350 degrees C. *Am J Sci* **278**, 838-860.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W. & Stetter, K. O. (1997). Pyrolobus fumarii , gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles* **1**, 14-21.

Bohnenstiehl, D. R., Dziak, R. P., Tolstoy, M., Fox, C. G. & Fowler, M. (2004). Temporal and spatial history of the 1999–2000 Endeavour Segment seismic series, Juan de Fuca Ridge. *Geochem Geophys Geosyst* **5**, Q09003.

Bonch-Osmolovskaya, E. A., Miroshnichenko, M. L., Slobodkin, A. I. & other authors (1999). Biodiversity of anaerobic lithotrophic prokaryotes in terrestrial hot springs of Kamchatka. *Microbiology* **68**, 343-351.

Boonyaratanakornkit, B., Miao, L. & Clark, D. (2007). Transcriptional responses of the deep-sea hyperthermophile Methanocaldococcus jannaschii under shifting extremes of temperature and pressure. *Extremophiles* **11**, 495-503.

Brock, T. D. & Gustafson, J. (1976). Ferric iron reduction by sulfur- and iron-oxidizing bacteria. *Applied Environmental Microbiolgy* **32**, 567-571.

Burggraf, S., Fricke, H., Neuner, A., Kristjansson, J., Rouvier, P., Mandelco, L., Woese, C. & Stetter, K. (1990a). Methanococcus igneus sp. nov., a novel hyperthermophilic methanogen from a shallow submarine hydrothermal system. *Systematic Applied Microbiolgy* **13**, 263-269.

Burggraf, S., Jannasch, H. W., Nicolaus, B. & Stetter, K. O. (1990b). Archaeoglobus profundus sp. nov., represents a new species within the sulfate-reducing arachaebacteria. *Systematic and Applied Microbiology* **13**, 5.

Butterfield, D. A., McDuff, R. E., Mottl, M. J., Lilley, M. D., Lupton, J. E. & Massoth, G. J. (1994). Gradients in the composition of hydrothermal fluids from the Endeavour segment vent field: Phase separation and brine loss. *J Geophys Res* **99**, 9561-9583.

Butterfield, D. A., Jonasson, I. R., Massoth, G. J. & other authors (1997). Seafloor eruptions and evolution of hydrothermal fluid chemistry. *Philosophical Transactions: Mathematical, Physical and Engineering Sciences* **355**, 369-386.

Butterfield, D. A., Roe, K. K., Lilley, M. D., Huber, J. A., Baross, J. A., Embley, R. W. & Massoth, G. J. (2004). Mixing, reaction and microbial activity in the sub-seafloor revealed by temporal and spatial variation in diffuse flow vents at Axial Volcano. In *The Subseafloor Biosphere at Mid-Ocean Ridges*, pp. 21. Edited by W. S. D. Wilcock, E. F. FeLong, D. S. Kelley, J. A. Baross & S. C. Cary. Washington, DC: American Geophysical Union.

CASM (1985). Hydrothermal vents on an Axis Seamount of the Juan de Fuca Ridge. *Nature* **313**, 212-214.

Chapelle, F. H., Haack, S. K., Adriaens, P., Henry, M. A. & Bradley, P. M. (1996). Comparison of Eh and H2 measurements for delineating redox processes in a contaminated aquifer. *Environmental Science & Technology* **30**, 3565-3569.

Chapelle, F. H., Vroblesky, D. A., Woodward, J. C. & Lovley, D. R. (1997). Practical considerations for measuring hydrogen concentrations in groundwater. *Environmental Science & Technology* **31**, 2873-2877.

Chapelle, F. H., O'Neill, K., Bradley, P. M., Methe, B. A., Ciufo, S. A., Knobel, L. L. & Lovley, D. R. (2002). A hydrogen-based subsurface microbial community dominated by methanogens. *Nature* **415**, 312-315.

Chave, J. & Leigh, E. G. (2002). A spatially explicit neutral model of diversity in tropical forests. *Theoretical Population Biology* **62**, 153-168.

Chave, J. (2004). Neutral theory and community ecology. *Ecology Letters* **7**, 241-253.

Chevaldonné, P. & Godfroy, A. (1997). Enumeration of microorganisms from deep-sea hydrothermal chimney samples. *FEMS Microbiology Letters* **146**, 211-216.

Childers, S. E. & Lovley, D. R. (2001). Differences in Fe(III) reduction in the hyperthermophilic archaeon, Pyrobaculum islandicum, versus mesophilic Fe(III)-reducing bacteria. *FEMS Microbiology Letters* **195**, 253-258.

Chou, C.-J., Shockley, K. R., Conners, S. B., Lewis, D. L., Comfort, D. A., Adams, M. W. W. & Kelly, R. M. (2007). Impact of substrate glycoside linkage and elemental sulfur on bioenergetics of and hydrogen production by the hyperthermophilic archaeon Pyrococcus furiosus. *Appl Environ Microbiol* **73**, 6842-6853.

Christensen, T. H., Bjerg, P. L., Banwart, S. A., Jakobsen, R., Heron, G. & Albrechtsen, H.-J. (2000). Characterization of redox conditions in groundwater contaminant plumes. *Journal of Contaminant Hydrology* **45**, 165-241.

Chyba, C. F. & Phillips, C. B. (2007). Europa. In *Planets and Life* pp. 388-423. Edited by W. T. Sullivan & J. A. Baross. New York Cambridge University Press

Clague, D. A., Caress, D. W., Thomas, H., Thompson, D., Calarco, M., Holden, J. & Butterfield, D. (2008). Abundance and distribution of hydrothermal chimneys and mounds on the Endeavour ridge determined by 1-m resolution AUV multibeam mapping surveys. *AGU Fall Meeting Abstracts*, B2079+.

Cordruwisch, R., Seitz, H. J. & Conrad, R. (1988). The capacity of hydrogenotrophic anaerobic-bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron-acceptor. *Archives of Microbiology* **149**, 350-357.

Corliss, J. B., Dymond, J., Gordon, L. I. & other authors (1979). Submarine thermal springs on the Galapagos rift. *Science* **203**, 1073-1083.

Corliss, J. B., Baross, J. A. & Hoffmann, S. E. (1981). An hypothesis concerning the relationship between submarine hotsprings and the origin of life on Earth. *Oceanologica Acta* **4**, 59-69.

Crocetti, G., Murto, M. & Björnsson, L. (2006). An update and optimisation of oligonucleotide probes targeting methanogenic Archaea for use in fluorescence in situ hybridisation (FISH). *Journal of Microbiological Methods* **65**, 194-201.

Daley, R. J. & Hobbie, J. E. (1975). Direct Counts of Aquatic Bacteria by a Modified Epifluorescence Technique. *Limnology and Oceanography* **20**, 875-882.

Daniel, R. M., Holden, J. F., Truter, J., Cowan, D. A. & Eckert, R. v. (2004). The stability of biomolecules and the implications for life at high temperatures. In *The Subsurface Biosphere at Mid-Ocean Ridges*, pp. 25-40. Edited by W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross & S. C. Cary. Washington, DC: American Geophysical Union.

Darwin, C. (1859). *The origin of species by means of natural selection or the preservation of favored races in the struggle for life*. London, U.K.: Murray.

De Rosa, M., Morana, A., Riccio, A., Gambacorta, A., Trincone, A. & Incani, O. (1994). Lipids of the Archaea: A new tool for bioelectronic. *Biosensors & bioelectronics* **9**, 669-675.

Delaney, J. R., Robigou, V., McDuff, R. E. & Tivey, M. K. (1992). Geology of a vigorous hydrothermal system on the Endeavour Segment, Juan de Fuca Ridge. *J Geophys Res* **97**, 19663-19682.

Delaney, J. R., Kelley, D. S., Mathez, E. A., Yoerger, D. R., Baross, J., Schrenk, M. O., Tivey, M. K., Kaye, J. & Robigou, V. (2001). "Edifice Rex" sulfide recovery project: Analysis of submarine hydrothermal, microbial habitat. *Eos, Transactions American Geophysical Union* **82**, 67.

Deming, J. W. & Baross, J. A. (1993). Deep-sea smokers: Windows to a subsurface biosphere? *Geochimica et Cosmochimica Acta* **57**, 3219-3230.

Deppenmeier, U. & Müller, V. (2008). Life close to the thermodynamic limit: How methanogenic archaea conserve energy. In *Bioenergetics: Energy Conservation and Conversion*, pp. 123-152. Edited by G. Schäfer & H. S. Penefsky. Berlin, Germany: Springer-Verlag.

DiRuggiero, J., Santangelo, N., Nackerdien, Z., Ravel, J. & Robb, F. (1997). Repair of extensive ionizing-radiation DNA damage at 95 degrees C in the hyperthermophilic archaeon Pyrococcus furiosus. *J Bacteriol* **179**, 4643-4645.

Dziak, R. P. & Fox, C. G. (1999). The January 1998 earthquake swarm at Axial Volcano, Juan de Fuca Ridge: Hydroacoustic evidence of seafloor volcanic activity. *Geophys Res Lett* **26**, 3429-3432.

Ehrenberg, C. G. (1838). *Die Infusionsthierchen als vollkommene Organismen (The infusion animalcules as perfect organisms)*. Leipzig, Germany: Voss.

Elkins, J. G., Podar, M., Graham, D. E. & other authors (2008). A korarchaeal genome reveals insights into the evolution of the Archaea. *Proceedings of the National Academy of Sciences* **105**, 8102-8107.

Ellis, E. A. (2006). Solutions to the problem of substitution of ERL 4221 for vinyl cyclohexene dioxide in spurr low viscosity embedding formulations. *Microscopy Today* **14**, 32-33.

Embley, R. W., Murphy, K. M. & Fox, C. G. (1990). High-Resolution Studies of the Summit of Axial Volcano. *J Geophys Res* **95**, 12785-12812.

Embley, R. W., Chadwick, W. W., Jr., Clague, D. & Stakes, D. (1999). 1998 eruption of Axial Volcano: Multibeam anomalies and seafloor observations. *Geophys Res Lett* **26**, 3425-3428.

Erauso, G., Reysenbach, A.-L., Godfroy, A. & other authors (1993). Pyrococcus abyssi sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Archives of Microbiology* **160**, 338-349.

Fardeau, M., Peillex, J. & Belaïch, J. (1987). Energetics of the growth of Methanobacterium thermoautotrophicum and Methanococcus thermolithotrophicus on ammonium chloride and dinitrogen. *Archives of Microbiology* **148**, 128-131.

Feinberg, L. F. & Holden, J. F. (2006). Characterization of dissimilatory Fe(III) versus NO3- reduction in the hyperthermophilic archaeon Pyrobaculum aerophilum. *J Bacteriol* **188**, 525-531.

Feinberg, L. F., Srikanth, R., Vachet, R. W. & Holden, J. F. (2008). Constraints on anaerobic respiration in the hyperthermophilic archaea Pyrobaculum islandicum and Pyrobaculum aerophilum. *Appl Environ Microbiol* **74**, 396-402.

Fiala, G. & Stetter, K. O. (1986). Pyrococcus furiosus sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. *Archives of Microbiology* **145**, 56-61.

Fornari, D. J. & Embley, R. W. (1995). Tectonic and volcanic controls on hydrothermal processes at the mid-ocean ridge: An overview based on near-bottom and submersible studies. In *Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions*, pp. 1-46. Edited by S. E. Humphris, R. A. Ziernberg, L. S. Mullineaux & R. E. Thomson. Washington, DC: American Geophysical Union - Geophysical Monograph Series.

Forterre, P., Bergerat, A. & Lopex-Garcia, P. (1996). The unique DNA topology and DNA topoisomerases of hyperthermophilic archaea. *FEMS Microbiology Reviews* **18**, 237-248.

Gambacorta, A., Gliozzi, A. & Rosa, M. (1995). Archaeal lipids and their biotechnological applications. *World Journal of Microbiology and Biotechnology* **11**, 115-131.

Ganoza, M. C., Kiel, M. C. & Aoki, H. (2002). Evolutionary conservation of reactions in translation. *Microbiol Mol Biol Rev* **66**, 460-485.

Gao, B. & Gupta, R. S. (2007). Phylogenomic analysis of proteins that are distinctive of Archaeaand its main subgroups and the origin of methanogenesis: BioMed Central Ltd.

German, C. R. (2004). Hydrothermal exploration and astrobiology: oases for life in distant oceans? *International Journal of Astrobiology* **3**, 81-95.

Girguis, P. R. & Lee, R. W. (2006). Thermal preference and tolerance of alvinellids. *Science* **312**, 231.

Glickson, D. A., Kelley, D. S. & Delaney, J. R. (2007). Geology and hydrothermal evolution of the Mothra Hydrothermal Field, Endeavour Segment, Juan de Fuca Ridge. *Geochem Geophys Geosyst* **8**, Q06010.

Godfroy, A., Lesongeur, F., Raguenes, G., Querellou, J., Antoine, E., Meunier, J.-R., Guezennec, J. & Barbier, G. (1997). Thermococcus hydrothermalis sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **47**, 622-626.

Gold, T. (1992). The deep, hot biosphere. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6045-6049.

Gold, T. (1999). *The Deep Hot Biosphere*. New York: Copernicus.

González, J. M., Masuchi, Y., Robb, F. T., Ammerman, J. W., Maeder, D. L., Yanagibayashi, M., Tamaoka, J. & Kato, C. (1998). Pyrococcus horikoshii sp. nov., a hyperthermophilic archaeon isolated from a hydrothermal vent at the Okinawa Trough. *Extremophiles* **2**, 123-130.

Grayling, R. A., Sandman, K. & Reeve, J. N. (1996). Histones and chromatin structure in hyperthermophilic archaea. *FEMS Microbiology Reviews* **18**, 203-213.

Greene, A. C., Patel, B. K. C. & Sheety, A. J. (1997). Deferribacter thermophilus gen. nov., sp. nov., a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. *Int J Syst Bacteriol* **47**, 505-509.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Roßnagel, P., Burggraf, S., Huber, H. & Stetter, K. O. (1996). Ferroglobus placidus gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe2+ at neutral pH under anoxic conditions. *Archives of Microbiology* **166**, 308-314.

Haldane, J. B. S. (1929). The origin of life. *Rationalist Annual* **148**, 3-10.

Hales, B., Edwards, C., Ritchie, D., Hall, G., Pickup, R. & Saunders, J. (1996). Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62**, 668-675.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95-98.

Hammond, S. R. (1990). Relationships between lava types, seafloor morphology, and the occurance of hydrothermal venting in the ASHES vent field of Axial Volcano. *J Geophys Res* **95**, 12875-12893.

Hansen, C., Esposito, L., Stewart, A. I. F., Colwell, J., Hendrix, A., Pryor, W., Shemansky, D. & West, R. (2006). Enceladus' water vapor plume. *Science* **311**, 1422-1425.

Harmsen, H., Prieur, D. & Jeanthon, C. (1997). Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridization and enrichment culture of thermophilic subpopulations. *Appl Environ Microbiol* **63**, 2876-2883.

Hedrick, D. B., Pledger, R. D., White, D. C. & Baross, J. A. (1992). In situ microbial ecology of hydrothermal vent sediments. *FEMS Microbiology Letters* **101**, 1-10.

Higashi, Y., Sunamura, M., Kitamura, K., Nakamura, K. i., Kurusu, Y., Ishibashi, J. i., Urabe, T. & Maruyama, A. (2004). Microbial diversity in hydrothermal surface to subsurface environments of Suiyo Seamount, Izu-Bonin Arc, using a catheter-type in situ growth chamber. *FEMS Microbiology Ecology* **47**, 327-336.

Hoehler, T. M., Alperin, M. J., Albert, D. B. & Martens, C. S. (1998). Thermodynamic control on hydrogen concentrations in anoxic sediments. *Geochimica et Cosmochimica Acta* **62**, 1745-1756.

Hoehler, T. M. (2004). Biological energy requirements as quantitative boundary conditions for life in the subsurface. *Geobiology* **2**, 205-215.

Hoek, J., Banta, A., Hubler, F. & Reysenbach, A.-L. (2003). Microbial diversity of a sulphide spire located in the Edmond deep-sea hydrothermal vent field on the Central Indian Ridge. *Geobiology* **1**, 119-127.

Holden, J. F. & Baross, J. A. (1995). Enhanced thermotolerance by hydrostatic pressure in the deep-sea hyperthermophile Pyrococcus strain ES4. *FEMS Microbiology Ecology* **18**, 27-34.

Holden, J. F., Summit, M. & Baross, J. A. (1998). Thermophilic and hyperthermophilic microorganisms in 3-30 C hydrothermal fluids following a deep-sea volcanic eruption. *FEMS Microbiology Ecology* **25**, 33-41.

Holden, J. F., Takai, K., Summit, M., Bolton, S., Zyskowski, J. & Baross, J. A. (2001). Diversity among three novel groups of hyperthermophilic deep-sea Thermococcus species from three sites in the northeastern Pacific Ocean. *FEMS Microbiology Ecology* **36**, 51-60.

Holden, J. F. & Adams, M. W. W. (2003). Microbe-metal interactions in marine hydrothermal environments. *Current Opinion in Chemical Biology* **7**, 160-165.

Holden, J. F. & Daniel, R. M. (2004). The upper temperature limit of life based on hyperthermophilic culture experiments and field observations. In *The Subsurface Biosphere at Mid-Ocean Ridges*, pp. 13-24. Edited by W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross & S. C. Cary. Washington DC: American Geophysical Union.

Holden, J. F. (2009). Extremophiles: Hot environments. In *Encyclopedia of Microbiology*, pp. 127-146. Edited by M. Schaechter. Elsevier: Oxford.

Holden, J. F., Menon, A. L. & Adams, M. W. W. (2011). Hyperthermophile-metal interactions in hydrothermal environments. In *Microbial Metal and Metalloid Metabolism: Advances and Applications*. Edited by J. F. Stolz & R. S. Oremland. Washington D.C.: American Society of Microbiology.

Holland, M. E. & Baross, J. A. (2003). Limits to life in hydrothermal systems. In *Energy and Mass Transfer in Marine Hydrothermal Systems*, pp. 235-246. Edited by P. E. Halbach, V. Tunnicliffe & J. R. Hein. Berlin: Dahlem University Press.

Holland, M. E., Baross, J. A. & Holden, J. F. (2004). Illuminating subseafloor ecosystems using microbial tracers. In *The Subsurface Biosphere at Mid-Ocean Ridges*, pp. 291-304. Edited by W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross & S. C. Cary. Washington DC: American Geophysical Union.

Holm, N. G. & Andersson, E. (2005). Hydrothermal simulation experiments as a tool for studies of the origin of life on earth and other terrestrial planets: A review. *Astrobiology* **5**, 444-460.

Hubbell, S. P. (1997). A unified theory of biogeography and relative species abundance and its application to tropical rain forests and coral reefs. *Coral Reefs* **16**, S9-S21.

Hubbell, S. P. (2001). *The Unified Neutral Theory of Biodiversity and Biogeography*. Princeton, NJ: Princeton University Press.

Hubbell, S. P. (2006). Neutral theory and the evolution of ecological equivalence. *Ecology* **87**, 1387-1398.

Huber, H. & Stetter, K. O. (1998). Hyperthermophiles and their possible potential in biotechnology. *Journal of Biotechnology* **64**, 39-52.

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C. & Stetter, K. O. (2002a). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* **417**, 63-67.

Huber, J. A., Butterfield, D. A. & Baross, J. A. (2002b). Temporal changes in archaeal diversity and chemistry in a mid-ocean ridge subseafloor habitat. *Appl Environ Microbiol* **68**, 1585-1594.

Huber, J. A., Mark Welch, D. B., Morrison, H. G., Huse, S. M., Neal, P. R., Butterfield, D. A. & Sogin, M. L. (2007). Microbial population structures in the deep marine biosphere. *Science* **318**, 97-100.

Huber, J. A. & Holden, J. F. (2008). Modeling the impact of diffuse vent microorganisms along mid-ocean ridges and flanks. In *Magma to Microbe, Modeling Hydrothermal Processes at Oceanic Spreading Centers*, pp. 215-232. Edited by R. P. Lowell, J. S. Seewald, A. Metaxias & M. R. Perfit. Washington, D.C.: American Geophysical Union.

Huber, J. A., Merkel, A., Holden, J. F., Lilley, M. D. & Butterfield, D. A. (2009). Molecular diversity and activity of methanogens in the subseafloor at deep-sea hydrothermal vents of the Pacific Ocean *American Geophysical Union, Fall Meeting Abstracts*, OS12A-08.

Hutchinson, G. E. (1965). The niche: an abstractly inhabited hypervolume. In *The Ecological Theatre and the Evolutionary Play*, pp. 26-78: Yale University Press.

Jakobsen, R., Albrechtsen, H.-J., Rasmussen, M., Bay, H., Bjerg, P. L. & Christensen, T. H. (1998). H2 concentrations in a landfill leachate plume (Grindsted, Denmark): In situ energetics of terminal electron acceptor processes. *Environmental Science & Technology* **32**, 2142-2148.

Jannasch, H. W. (1995). Microbial interactions with hydrothermal fluids. In *Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions*, pp. 273-296. Edited by S. E. Humphries, R. Zierenberg, A, L. S. Mullineaux & R. E. Thomson. Washington, DC: American Geophysical Union.

Jeanthon, C., L'Haridon, S., Reysenbach, A. L., Vernet, M., Messner, P., Sleytr, U. B. & Prieur, D. (1998). Methanococcus infernus sp. nov., a novel hyperthermophilic lithotrophic methanogen isolated from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **48**, 913-919.

Jeanthon, C., L'Haridon, S., Pradel, N. & Prieur, D. (1999a). Rapid identification of hyperthermophilic methanococci isolated from deep-sea hydrothermal vents. *Int J Syst Bacteriol* **49**, 591-594.

Jeanthon, C., L'Haridon, S., Reysenbach, A.-L., Corre, E., Vernet, M., Messner, P., Sleytr, U. B. & Prieur, D. (1999b). Methanococcus vulcanius sp. nov., a novel hyperthermophilic methanogen isolated from East Pacific Rise, and identification of Methanococcus sp. DSM 4213Tas Methanococcus fervens sp. nov. *Int J Syst Bacteriol* **49**, 583-589.

Jeanthon, C. (2000). Molecular ecology of hydrothermal vent microbial communities. *Antonie van Leeuwenhoek* **77**, 117-133.

Johnson, H. P., Hutnak, M., Dziak, R. P., Fox, C. G., Urcuyo, I., Cowen, J. P., Nabelek, J. & Fisher, C. (2000). Earthquake-induced changes in a hydrothermal system on the Juan de Fuca mid-ocean ridge. *Nature* **407**, 174-177.

Johnson, M. R., Conners, S. B., Montero, C. I., Chou, C. J., Shockley, K. R. & Kelly, R. M. (2006). The Thermotoga maritima phenotype Is impacted by syntrophic interaction with Methanococcus jannaschii in hyperthermophilic coculture. *Appl Environ Microbiol* **72**, 811-818.

Jones, W. J., Leigh, J. A., Mayer, F., Woese, C. R. & Wolfe, R. S. (1983a). Methanococcus jannaschii sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Archives of Microbiology* **136**, 254-261.

Jones, W. J., Paynter, M. J. B. & Gupta, R. (1983b). Characterization of Methanococcus maripaludis sp. nov., a new methanogen isolated from salt marsh sediment. *Archives of Microbiology* **135**, 91-97.

Jones, W. J., Stugard, C. E. & Jannasch, H. W. (1989). Comparison of thermophilic methanogens from submarine hydrothermal vents. *Archives of Microbiology* **151**, 314-318.

Juniper, S. K., Cambon, M. A., Lesongeur, F. & Barbier, G. (2001). Extraction and purification of DNA from organic rich subsurface sediments (ODP Leg 169S). *Marine Geology* **174**, 241-247.

Karl, D., Michaels, A., Bergman, B. & other authors (2002). Dinitrogen fixation in the world's oceans. *Biogeochemistry* **57-58**, 47-98.

Karl, D. M. (1995). Ecology of free-living, hydrothermal vent microbial communities. In *The Microbiology of Deep-Sea Hydrothermal Vents*, pp. 1050-1056. Edited by D. M. Karl. New York: CRC Press.

Kashefi, K. & Lovley, D. R. (2000). Reduction of Fe(III), Mn(IV), and toxic metals at 100C by Pyrobaculum islandicum. *Appl Environ Microbiol* **66**, 1050-1056.

Kashefi, K., Tor, J. M., Holmes, D. E., Gaw Van Praagh, C. V., Reysenbach, A. L. & Lovley, D. R. (2002). Geoglobus ahangari gen. nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor. *Int J Syst Evol Microbiol* **52**, 719-728.

Kashefi, K. & Lovley, D. R. (2003). Extending the upper temperature limit for life. *Science* **301**, 934.

Kashefi, K., Shelobolina, E. S., Elliott, W. C. & Lovley, D. R. (2008). Growth of Thermophilic and Hyperthermophilic Fe(III)-Reducing Microorganisms on a Ferruginous Smectite as the Sole Electron Acceptor. *Appl Environ Microbiol* **74**, 251-258.

Kelley, D. S., Delaney, J. R., Lilley, M. D. & Butterfield, D. A. (2001a). Vent field distribution and evolution along the Endeavour Segment, Juan de Fuca Ridge. *AGU Fall Meeting Abstracts*, B439+.

Kelley, D. S., Delaney, J. R. & Yoerger, D. R. (2001b). Geology and venting characteristics of the Mothra hydrothermal field, Endeavour segment, Juan de Fuca Ridge. *Geology*, 959-962.

Kelley, D. S., Baross, J. A. & Delaney, J. R. (2002). Volcanoes, fluids, and life at mid-ocean ridge spreading centers *Annual Review of Earth and Planetary Sciences* **30**, 385-491.

Kormas, K. A., Tivey, M. K., Von Damm, K. & Teske, A. (2006). Bacterial and archaeal phylotypes associated with distinct mineralogical layers of a white smoker spire from a deep-sea hydrothermal vent site (9°N, East Pacific Rise). *Environmental Microbiology* **8**, 909-920.

Koval, S. F. & Jarrell, K. F. (1987). Ultrastructure and biochemistry of the cell wall of Methanococcus voltae. *J Bacteriol* **169**, 1298-1306.

Kristall, B., Kelley, D. S., Hannington, M. D. & Delaney, J. R. (2006). Growth history of a diffusely venting sulfide structure from the Juan de Fuca Ridge: A petrological and geochemical study. *Geochem Geophys Geosyst* **7**, Q07001.

Kurr, M., Huber, R., König, H., Jannasch, H. W., Fricke, H., Trincone, A., Kristjansson, J. K. & Stetter, K. O. (1991). Methanopyrus kandleri, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Archives of Microbiology* **156**, 239-247.

L'Haridon, S., Reysenbach, A.-L., Banta, A., Messner, P., Schumann, P., Stackebrandt, E. & Jeanthon, C. (2003). Methanocaldococcus indicus sp. nov., a novel hyperthermophilic methanogen isolated from the Central Indian Ridge. *Int J Syst Evol Microbiol* **53**, 1931-1935.

Larkin, M. A., Blackshields, G., Brown, N. P. & other authors (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.

Lauerer, G., Kristjansson, J., Langworthy, T., Koenig, H. & Stetter, K. (1986). Methanothermus sociabilis sp. nov., a second species within the Methanothermaceae growing at 97 degree C. . *Systematic and Applied Microbiology [SYST APPL MICROBIOL]* **8**, 100-105.

Lilley, M. D., Butterfield, D. A., Olson, E. J., Lupton, J. E., Macko, S. A. & McDuff, R. E. (1993). Anomalous CH4 and NH4+ concentrations at an unsedimented mid-ocean-ridge hydrothermal system. *Nature* **364**, 45-47.

Lilley, M. D., Butterfield, D. A., Lupton, J. E. & Olson, E. J. (2003). Magmatic events can produce rapid changes in hydrothermal vent chemistry. *Nature* **422**, 878-881.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* **362**, 709-715.

Lipp, J. S., Morono, Y., Inagaki, F. & Hinrichs, K.-U. (2008). Significant contribution of Archaea to extant biomass in marine subsurface sediments. *Nature* **454**, 991-994.

Liu, S. V., Zhou, J., Zhang, C., Cole, D. R., Gajdarziska-Josifovska, M. & Phelps, T. J. (1997). Thermophilic Fe(III)-reducing bacteria from the deep subsurface: The evolutionary implications. *Science* **277**, 1106-1109.

Lovley, D. R. & Phillips, E. J. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and environmental microbiology* **51**, 683-689.

Lovley, D. R. & Goodwin, S. (1988). Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochimica et Cosmochimica Acta* **52**, 2993-3003.

Lovley, D. R., Chapelle, F. H. & Woodward, J. C. (1994). Use of dissolved H2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. *Environmental Science & Technology* **28**, 1205-1210.

Lovley, D. R. (2000a). Dissimilatory Fe(III) and Mn(IV)-reducing prokaryotes. In *The Prokaryotes*. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.

Lovley, D. R. (2000b). Fe(III) and Mn(IV) reduction. In *Enviornmental Microbe-Metal Interactions*, pp. 3-30. Edited by D. R. Lovley. Washington, DC: American Society for Microbiology.

Lovley, D. R. (2002). Dissimilatory Metal Reduction: from Early Life to Bioremediation. *ASM News* **68**, 231-237.

Lu, X.-X., Tao, S., Bosma, T. & Gerritse, J. (2001). Characteristic hydrogen concentrations for various redoc processes in batch study. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering* **36**, 1725 - 1734.

Luther, G. W., Rozan, T. F., Taillefert, M., Nuzzio, D. B., Di Meo, C., Shank, T. M., Lutz, R. A. & Cary, S. C. (2001). Chemical speciation drives hydrothermal vent ecology. *Nature* **410**, 813-816.

Luton, P. E., Wayne, J. M., Sharp, R. J. & Riley, P. W. (2002). The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* **148**, 3521-3530.

Lynd, L. R., Weimer, P. J., van Zyl, W. H. & Pretorius, I. S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol Mol Biol Rev* **66**, 506-577.

MacArthur, R. H. & Wilson, E. O. (1963). An equilibrium theory of island biogography. *Evolution* **17**, 373-387.

MacArthur, R. H. & Wilson, E. O. (1967). *The Theory of Island Biogoegraphy*. Princeton, New Jersey: Princeton University Press.

Marscarelli, A. L. (2009). Low Life. *Nature* **459**, 770-773.

Marteinsson, V. T., Birrien, J.-L., Reysenbach, A.-L., Vernet, M., Marie, D., Gambacorta, A., Messner, P., Sleytr, U. B. & Prieur, D. (1999). Thermococcus barophilus sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **49**, 351-359.

Martin, W., Baross, J., Kelley, D. & Russell, M. J. (2008). Hydrothermal vents and the origin of life. *Nat Rev Micro* **6**, 805-814.

McCliment, E. A., Voglesonger, K. M., O'Day, P. A., Dunn, E. E., Holloway, J. R. & Cary, S. C. (2006). Colonization of nascent, deep-sea hydrothermal vents by a novel Archaeal and Nanoarchaeal assemblage. *Environmental Microbiology* **8**, 114-125.

McCollom, T. M. & Shock, E. L. (1997). Geochemical constraints on chemolithoautotrophic metabolism by microorganisms in seafloor hydrothermal systems. *Geochimica et Cosmochimica Acta* **61**, 4375-4391.

McKay, D. S., Gibson, E. K., Thomas-Keprta, K. L., Vali, H., Romanek, C. S., Clemett, S. J., Chillier, X. D. F., Maechling, C. R. & Zare, R. N. (1996). Search for past life on Mars: Possible relic biogenic activity in Martian meteorite *Science* **273**, 924-930.

Mehta, M. P., Butterfield, D. A. & Baross, J. A. (2003). Phylogenetic diversity of nitrogenase (nifH) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. *Appl Environ Microbiol* **69**, 960-970.

Mehta, M. P. & Baross, J. A. (2006). Nitrogen fixation at 92 degrees celsius by a hydrothermal vent archaeon. *Science* **314**, 1783-1786.

Miller, J. F., Shah, N. N., Nelson, C. M., Ludlow, J. M. & Clark, D. S. (1988). Pressure and temperature effects on growth and methane production of the extreme thermophile Methanococcus jannaschii. *Appl Environ Microbiol* **54**, 3039-3042.

Miller, J. F., Nelson, C. M., Ludlow, J. M., Shah, N. N. & Clark, D. S. (1989). High pressure-temperature bioreactor: Assays of thermostable hydrogenase with fiber optics. *Biotechnology and Bioengineering* **34**, 1015-1021.

Miller, S. L. (1953). A production of amino acids under possible primitive Earth conditions. *Science* **117**, 528-529.

Miroshnichenko, M. L., Gongadze, G. M., Rainey, F. A., Kostyukova, A. S., Lysenko, A. M., Chernyh, N. A. & Bonch-Osmolovskaya, E. A. (1998). Thermococcus gorgonarius sp. nov. and Thermococcus pacificus sp. nov.: heterotrophic extremely thermophilic archaea from New Zealand submarine hot vents. *Int J Syst Bacteriol* **48**, 23-29.

Morita, R. (1997). *Bacteria in Oligotrophic Environments: Starvation-Survival Lifestyle*. New York: Champman and Hall.

Nakagawa, S., Takai, K., Inagaki, F. & other authors (2005). Variability in microbial community and venting chemistry in a sediment-hosted backarc hydrothermal system: Impacts of subseafloor phase-separation. *FEMS Microbiology Ecology* **54**, 141-155.

Nakagawa, T., Takai, K., Suzuki, Y., Hirayama, H., Konno, U., Tsunogai, U. & Horikoshi, K. (2006). Geomicrobiological exploration and characterization of a novel deep-sea hydrothermal system at the TOTO caldera in the Mariana Volcanic Arc. *Environmental Microbiology* **8**, 37-49.

Nazina, T. N., Ivanova, A. E., Goulbeva, O. V., Ibatullin, R. R., Belyaev, S. S. & Ivanov, M. V. (1995). Occurence of sulfate- and iron-reducing bacteria in stratal waters of the Romashkinskoe oil field. *Microbiology* **64**, 203-208.

Nealson, K. H., Inagaki, F. & Takai, K. (2005). Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): do they exist and why should we care? *Trends in Microbiology* **13**, 405-410.

Nercessian, O., Fouquet, Y., Pierre, C., Prieur, D. & Jeanthon, C. (2005). Diversity of Bacteria and Archaea associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. *Environmental Microbiology* **7**, 698-714.

Newberry, C. J., Webster, G., Cragg, B. A., Parkes, R. J., Weightman, A. J. & Fry, J. C. (2004). Diversity of prokaryotes and methanogenesis in deep subsurface sediments from the Nankai Trough, Ocean Drilling Program Leg 190. *Environmental Microbiology* **6**, 274-287.

Nisbet, E. G. & Sleep, N. H. (2001). The habitat and nature of early life. *Nature* **409**, 1083-1091.

O'Malley, M. A. (2007). The nineteenth century roots of 'everything is everywhere'. *Nature Reviews Microbiology* **5**, 647-651.

Olsen, G. J., Woese, C. R. & Overbeek, R. (1994). The winds of (evolutionary) change: breathing new life into microbiology. *Journal of Bacteriology [J BACTERIOL]* **176**, 1-6.

Oparin, A. (1952). *The Origin of Life*. New York: Dover.

Orphan, V. J., House, C. H., Hinrichs, K.-U., McKeegan, K. D. & DeLong, E. F. (2001). Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**, 484-487.

Oslowski, D. M., Jung, J. H., Seo, D. H., Park, C. S. & Holden, J. F. (*submitted*). Hydrogen production from α-1,4 and β-1,4 linked saccharides by deep-sea hyperthermophilic archaea. *Applied and environmental microbiology*.

Pagé, A., Tivey, M. K., Stakes, D. S. & Reysenbach, A. L. (2008). Temporal and spatial archaeal colonization of hydrothermal vent deposits. *Environmental Microbiology* **10**, 874-884.

Patel, G. B. & Sprott, G. D. (1999). Archaeobacterial ether lipid liposomes (Archaeosomes) as novel vaccine and drug delivery systems. *Critical Reviews in Biotechnology* **19**, 317 - 357.

Pereira, S. L., Grayling, R. A., Lurz, R. & Reeve, J. N. (1997). Archaeal nucleosomes. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 12633-12637.

Perner, M., Kuever, J., Seifert, R., Pape, T., Koschinsky, A., Schmidt, K., Strauss, H. & Imhoff, J. F. (2007). The influence of ultramafic rocks on microbial communities at the Logatchev hydrothermal field, located 15°N on the Mid-Atlantic Ridge. *FEMS Microbiology Ecology* **61**, 97-109.

Petsko, G. A. (2001). [34] Structural basis of thermostability in hyperthermophilic proteins, or "There's more than one way to skin a cat". In *Methods in Enzymology*, pp. 469-478. Edited by R. M. K. Michael W. W. Adams: Academic Press.

Pledger, R. & Baross, J. (1989). Characterization of an extremely thermophilic archaebacterium isolated from a black smoker polychaete (Paralvinella sp.) at the Juan de Fuca Ridge. *Systematic and Applied Microbiology* **12**, 249-256.

Pley, U., Schipka, J., Gambacorta, A., Jannasch, H., Fricke, H., Rachel, R. & Stetter, K. (1991). Pyrodictium abyssi sp. nov. represents a novel heterotrophic marine archaeal hyperthermophile growing at 110 degree C. *Systematic and Applied Microbiology* **14**, 245-253.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* **35**, 7188-7196.

Reysenbach, A. L., Giver, L. J., Wickham, G. S. & Pace, N. R. (1992). Differential amplification of rRNA genes by polymerase chain reaction. *Appl Environ Microbiol* **58**, 3417-3418.

Robigou, V., Delaney, J. R. & Stakes, D. S. (1993). Large massive sulfide deposits in a newly discovered active hydrothermal system, The High Rise Field, Endeavour Segment, Juan De Fuca Ridge. *Geophys Res Lett* **20**, 1887-1890.

Roussel, E. G., Bonavita, M.-A. C., Querellou, J., Cragg, B. A., Webster, G., Prieur, D. & Parkes, R. J. (2008). Extending the sub-sea-floor biosphere. *Science* **320**, 1046-.

Roy, R., Mukund, S., Schut, G. J., Dunn, D. M., Weiss, R. & Adams, M. W. W. (1999). Purification and molecular characterization of the tungsten-containing formaldehyde ferredoxin oxidoreductase from the hyperthermophilic archaeon Pyrococcus furiosus: The third of a putative five-member tungstoenzyme family. *J Bacteriol* **181**, 1171-1180.

Rusch, A. & Amend, J. P. (2004). Order-specific 16S rRNA-targeted oligonucleotide probes for (hyper)thermophilic archaea and bacteria. *Extremophiles* **8**, 357-366.

Schidlowski, M., Hayes, J. M. & Kaplan, I. R. (1988). A 3800-million-year isotopic record of life from carbon in sedimentary rocks. *Nature* **333**, 313-318.

Schiraldi, C. & De Rosa, M. (2002). The production of biocatalysts and biomolecules from extremophiles. *Trends in Biotechnology* **20**, 515-521.

Schönheit, P. & Schäfer, T. (1995). Metabolism of hyperthermophiles. *World Journal of Microbiology and Biotechnology* **11**, 26-57.

Schrenk, M. O., Kelley, D. S., Delaney, J. R. & Baross, J. A. (2003). Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Appl Environ Microbiol* **69**, 3580-3592.

Schrenk, M. O., Holden, J. F. & Baross, J. A. (2008). Magma-to-microbe networks in the context of sulfide hosted microbial ecosystems. In *Magma to Microbe: Modeling Hydrothermal Processes at Oceanic Spreading Centers*, pp. 233-258. Edited by Robert P. Lowell, Jeff Seewald, Anna Metaxas & M. Perfit: Geophysical Monograph Series, AGU.

Schrenk, M. O., Huber, J. A. & Edwards, K. J. (2010). Microbial provinces in the subseafloor. *Annual Review of Marine Science* **2**, 279-304.

Segerer, A. H., Burggraf, S., Fiala, G., Huber, G., Huber, R., Pley, U. & Stetter, K. O. (1993). Life in hot springs and hydrothermal vents. *Origins of Life and Evolution of Biospheres* **23**, 77-90.

Seyfried, W. E., Jr., Seewald, J. S., Berndt, M. E., Ding, K. & Foustoukos, D. I. (2003). Chemistry of hydrothermal vent fluids from the Main Endeavour Field, northern Juan de Fuca Ridge: Geochemical controls in the aftermath of June 1999 seismic events. *J Geophys Res* **108**, 2429.

Shock, E. L. (1992). Chemical environments of submarine hydrothermal systems. *Origins of Life and Evolution of Biospheres* **22**, 67-107.

Sleep, N. H., Zahnle, K. J., Kasting, J. F. & Morowitz, H. J. (1989). Annihilation of ecosystems by large asteroid impacts on the early Earth: Nature Publishing Group.

Slobodkin, A., Campbell, B., Cary, S. C., Bonch-Osmolovskaya, E. & Jeanthon, C. (2001). Evidence for the presence of thermophilic Fe(III)-reducing microorganisms in deep-sea hydrothermal vents at 13N (East Pacific Rise). *FEMS Microbiology Ecology* **36**, 235-243.

Slobodkin, A. I. & Wiegel, J. (1997). Fe(III) as an electron acceptor for H2 oxidation in thermophilic anaerobic enrichment cultures from geothermal areas. *Extremophiles* **1**, 106-109.

Slobodkin, A. I., Zavarzina, D. G., Sokolova, T. G. & Bonch-Osmolovskaya, E. A. (1999). Dissimilatory reduction of inorganic electron acceptors by thermophilic anaerobic prokaryotes. *Microbiology* **68**, 522-542.

Slobodkina, G. B., Kolganova, T. V., Querellou, J., Bonch-Osmolovskaya, E. A. & Slobodkin, A. I. (2009). Geoglobus acetivorans sp. nov., an iron(III)-reducing archaeon from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **59**, 2880-2883.

Sorensen, K. B., Lauer, A. & Teske, A. (2004). Archaeal phylotypes in a metal-rich and low-activity deep subsurface sediment of the Peru Basin, ODP Leg 201, Site 1231. *Geobiology* **2**, 151-161.

Sorensen, K. B. & Teske, A. (2006). Stratified communities of active archaea in deep marine subsurface sediments. *Appl Environ Microbiol* **72**, 4596-4603.

Sowers, K. R. (1995). Methanogenic archaea: an overview. In *Archaea: A Laboratory Manual (Methanogens)*, pp. 3-13. Edited by K. R. Sowers & H. J. Schreier. Plainview, New York: Cold Spring Harbor Press.

Sprott, G. D., Meloche, M. & Richards, J. C. (1991). Proportions of diether, macrocyclic diether, and tetraether lipids in Methanococcus jannaschii grown at different temperatures. *J Bacteriol* **173**, 3907-3910.

Stetter, K., Koenig, H. & Stackebrandt, E. (1983). Pyrodictium gen.nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105C. . *Systematic and Applied Microbiology* **4**, 535-551.

Stetter, K. O., Thomm, M., Winter, J. & other authors (1981). Methanothermus fervidus, sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring: Fischer.

Stetter, K. O., Fiala, G., Huber, R., Huber, G. & Segerer, A. (1986). Life above the boiling point of water? *Cellular and Molecular Life Sciences* **42**, 1187-1191.

Stetter, K. O. (1996). Hyperthermophilic procaryotes. *FEMS Microbiology Reviews* **18**, 149-158.

Stetter, K. O. (1999). Extremophiles and their adaptation to hot environments. *FEBS Letters* **452**, 22-25.

Straube, W. L., Deming, J. W., Somerville, C. C., Colwell, R. R. & Baross, J. A. (1990). Particulate DNA in smoker fluids: Evidence for existence of microbial populations in hot hydrothermal systems. *Appl Environ Microbiol* **56**, 1440-1447.

Summit, M. & Baross, J. A. (2001). A novel microbial habitat in the mid-ocean ridge subseafloor. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 2158-2163.

Takai, K. & Horikoshi, K. (1999). Genetic diversity of Archaea in deep-sea hydrothermal vent environments. *Genetics* **152**, 1285-1297.

Takai, K., Komatsu, T., Inagaki, F. & Horikoshi, K. (2001). Distribution of Archaea in a black smoker chimney structure. *Appl Environ Microbiol* **67**, 3618-3629.

Takai, K., Gamo, T., Tsunogai, U., Nakayama, N., Hirayama, H., Nealson, K. H. & Horikoshi, K. (2004a). Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field. *Extremophiles* **8**, 269-282.

Takai, K., Nealson, K. H. & Horikoshi, K. (2004b). Methanotorris formicicus sp. nov., a novel extremely thermophilic, methane-producing archaeon isolated from a black smoker chimney in the Central Indian Ridge. *Int J Syst Evol Microbiol* **54**, 1095-1100.

Takai, K., Nakagawa, S., Reysenbach, A.-L. & Hoek, J. (2006). Microbial ecology of mid-ocean ridges and back-arc basins. *Geophysical monograph* **166**, 185-213.

Takai, K., Nakamura, K., Toki, T. & other authors (2008a). Cell proliferation at 122°C and isotopically heavy CH4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proceedings of the National Academy of Sciences* **105**, 10949-10954.

Takai, K., Nunoura, T., Ishibashi, J.-i. & other authors (2008b). Variability in the microbial communities and hydrothermal fluid chemistry at the newly discovered Mariner hydrothermal field, southern Lau Basin. *J Geophys Res* **113**, G02031.

Takai, K., Nunoura, T., Horikoshi, K. & other authors (2009). Variability in microbial communities in black smoker chimneys at the NW caldera vent field, Brothers Volcano, Kermadec Arc. *Geomicrobiology Journal* **26**, 552 - 569.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596-1599.

Ter Meer, J., Gerritse, J., Mauro, C., Harkes, M. P. & Rijnaarts, H. H. M. (1999). Hydrogen as indicator for in-situ redox condition and dechlorination. In *Natural Attenuation of Environmental Contaminants*, pp. 1-125. Edited by A. Leeson, M. E. Kelley, H. S. Rifai & V. S. Magar. Columbus, OH: Battelle Press.

Teske, A. P. (2005). The deep subsurface biosphere is alive and well. *Trends in Microbiology* **13**, 402-404.

Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W. & Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Micro* **6**, 579-591.

Tijhuis, L., Loosdrecht, M. C. M. V. & Heijnen, J. J. (1993). A thermodynamically based correlation for maintenance gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnology and Bioengineering* **42**, 509-519.

Tivey, M. K., Humphris, S. E., Thompson, G., Hannington, M. D. & Rona, P. A. (1995). Deducing patterns of fluid flow and mixing within the TAG active hydrothermal mound using mineralogical and geochemical data. *J Geophys Res* **100**, 12527-12555.

Tivey, M. K. (2004). Enviornmental conditions within active seafloor vent structures: Sensitivity to vent luid composition and fluid flow. In *The Subseafloor Biosphere at Mid-Ocean Ridges*, pp. 137-152. Edited by W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross & S. C. Cary. Washington, DC: American Geophysical Union.

Tor, J. M., Kashefi, K. & Lovley, D. R. (2001). Acetate oxidation coupled to Fe(III) reduction in hyperthermophilic microorganisms. *Appl Environ Microbiol* **67**, 1363-1365.

Tor, J. M. & Lovley, D. R. (2001). Anaerobic degradation of aromatic compounds coupled to Fe(III) reduction by *Ferroglobus placidus*. *Environmental Microbiology* **3**, 281-287.

Tornabene, T. & Langworthy, T. (1979). Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. *Science* **203**, 51-53.

Valentine, D. L. (2007). Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat Rev Micro* **5**, 316-323.

Van Ark, E. M., Detrick, R. S., Canales, J. P. & other authors (2007). Seismic structure of the Endeavour Segment, Juan de Fuca Ridge: Correlations with seismicity and hydrothermal activity. *J Geophys Res* **112**, B02401.

Van Dover, C. L. (2000). *The Ecology of Deep-Sea Hydrothermal Vents*. New Jersey: Princeton University Press.

Vargas, M., Kashefi, K., Blunt-Harris, E. L. & Lovley, D. R. (1998). Microbiological evidence for Fe(III) reduction on early Earth. *Nature* **395**, 65-67.

Ver Eecke, H. C., Kelley, D. S. & Holden, J. F. (2009). Abundances of hyperthermophilic autotrophic Fe(III) oxide reducers and heterotrophs in hydrothermal sulfide chimneys from the northeastern Pacific Ocean. *Appl Environ Microbiol*, AEM.01462-01408.

Von Damm, K. (2004). Evolution of the hydrothermal system at East Pacific Rise 9°50'N: Geochemical evidence for changes in the upper oceanic crust. In *The Subseafloor Biosphere at Mid-Ocean Ridges*, pp. 245-268. Edited by W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross & S. C. Cary. Washington, DC: American Geophysical Union.

Von Damm, K. L. (1990). Seafloor hydrothermal activity: Black smoker chemistry and chimneys. *Annual Review of Earth and Planetary Sciences* **18**, 173-204.

Wang, F., Zhou, H., Meng, J. & other authors (2009). GeoChip-based analysis of metabolic diversity of microbial communities at the Juan de Fuca Ridge hydrothermal vent. *Proceedings of the National Academy of Sciences* **106**, 4840-4845.

Whitman, W. B., Coleman, D. C. & Wiebe, W. J. (1998). Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6578-6583.

Woese, C. R. & Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5088-5090.

Woese, C. R. (1987). Bacterial evolution. *Microbiological reviews Baltimore [MICROBIOL REV]* **51**, 221-271.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4576-4579.

Xu, Y. & Glansdorff, N. (2002). Was our ancestor a hyperthermophilic procaryote? *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **133**, 677-688.

Yang, Y. & McCarty, P. L. (1998). Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. *Environmental Science & Technology* **32**, 3591-3597.

Zhou, H., Li, J., Peng, X., Meng, J., Wang, F. & Ai, Y. (2009). Microbial diversity of a sulfide black smoker in main endeavour hydrothermal vent field, Juan de Fuca Ridge. *The Journal of Microbiology* **47**, 235-247.

Zillig, W. (1991). Comparative biochemistry of Archaea and Bacteria. *Current Opinion in Genetics & Development* **1**, 544-551.